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Interaction of Plasma Proteins with Surfaces

C. A. Fenstermaker, W. H. Grant, B. W. Morrissey, L. E. Smith, R. R. Stromberg

Polymers Division
Institute for Materials Research
National Bureau of Standards
Washington, D. C. 20234

March 22, 1974

Annual Report for Period

July 1, 1972 – November 30, 1973

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SUMMARY

The interaction of blood proteins with surfaces has been investigated with principal attention focused on those proteins that are either major constituents of blood plasma or are implicated as being important in the clotting process. Emphasis has been placed on molecular conformational changes occurring upon the interaction of such proteins with surfaces. Materials have been selected which provide an insight into the mechanisms of these interactions. Materials for future study will include those which have been used or show potential utility in surgical procedures.

The extension of adsorbed molecules of fibrinogen, albumin, and prothrombin on a number of selected materials was studied by ellipsometry. No change in extension was observed for a given surface over a time period during which adsorbance was found to increase. This indicates a lack of molecular rearrangement after initial adsorption. The equilibrium extension was found to increase with decreasing surface energy for the surfaces studied, although the amounts adsorbed from solutions of physiological concentrations remained approximately constant. These results indicate a dependence of conformation on surface energy.

Measurements of the bound fraction (fraction of carbonyl groups of an adsorbed molecule directly in contact with the surface) of these adsorbed blood proteins on a silica surface showed that approximately ten percent of the carbonyl groups were attached to the surface for prothrombin and serum albumin at all values of surface population for the solution concentrations studied. Fibrinogen showed an increasing bound fraction with increasing adsorbance. The fact that no change in bound fraction occurred upon prior cross-linking and denaturation of albumin implied that changes in conformation upon adsorption to the silica surface, if they occurred, were small. Computer simulation studies of the adsorption of lysozyme were found to be consistent with these findings. Studies of the bound fraction of serum albumin as a function of pD and ionic strength show that caution must be exercised in identifying the plateau value for an adsorption isotherm for a protein with a close-packed monolayer.

Techniques have been developed for use with infrared spectroscopy which take advantage of the unique biological properties of prothrombin and fibrinogen to study competitive interactions of these proteins during the process of adsorption, displacement, and desorption. Some results are given.

Radiotracer studies have been initiated for measurements of the rates of adsorption and desorption. Washing techniques for such investigations have been developed. Equilibrium results obtained by the radiotracer method have been found to compare favorably with those obtained by ellipsometry. Rates of adsorption for albumin on chrome and silica surfaces have been measured. An important artifact was observed in the attempted adsorption measurements using iodine labeled albumin on a platinum surface. Specific adsorption of iodine causes preferential adsorption of the labeled protein.

INTERACTION OF PLASMA PROTEINS WITH SURFACES

INTRODUCTION

At present, the surface-induced coagulation of blood is a critical factor in the design and application of most devices for use with the cardiovascular system. The original studies of Freund (1) in 1885, as well as much recent work, e.g. ref. (2,3), have shown that the clotting time of blood is dependent upon the materials with which it is in contact. The current cascade model (4) emphasizes the role of numerous proteins in an enzyme-substrate relationship and stresses the probability that the initial event of surface-induced coagulation involves an interaction between the surface and a protein. The possible effects of a given surface on a protein would include, among others, permanent or reversible adsorption with or without concomitant denaturation and preferential adsorption of specific proteins. It is, therefore, of great importance to elucidate the details of the interaction of blood proteins with surfaces, especially if one hopes to develop a rational approach to the selection of thromboresistant materials.

Investigations on the interaction of blood proteins with surfaces have been recently reviewed (5-8). These studies have attempted to correlate the extent of the coagulation of plasma or blood with such surface properties as surface free energy (2,9), roughness (10), surface charge (11), and zeta potential (12). With few exceptions (13), investigations of the specific interaction of an adsorbed protein on a surface have, in general, been carried out on dried, collapsed films. Hence, at present, very little information exists that would enable a specification of the state of an adsorbed protein in situ. The importance of this information is stressed by recent studies (14) showing no patterns of selective adsorption or depression of enzymatic activity of blood factors by a number of surfaces when investigated by analysis of the supernatent solutions.

There is a need for a detailed understanding of the major events occurring at the blood-implant interface in order to assess their consequences and thus provide a rational basis for the design of materials and test methods. To this end we have studied various aspects of the interaction of several blood proteins with suitable surfaces, utilizing methods which have proven successful with synthetic polymer systems. Our attention has been focused initially on those proteins that are either major constituents of blood plasma or are implicated as being important in the clotting process. The surfaces investigated are those concerned with materials which have been used or show potential utility in surgical procedures or those which provide an insight into the mechanisms of the interactions. It is anticipated

that this information will be of immediate value in the selection of materials for use in heart assist devices such as catheters, heart valves, blood oxygenators, etc.

The specific aspects of the protein surface interactions that we have been investigating can be separated into three general areas:

1) Aspects of the changes in the conformation of the protein upon interaction with a surface. Specifically we are studying the changes in the average dimensions of the protein molecules as they interact with a surface as well as the number of contacts the molecule makes with the surface.

2) Measurement of the rates and amount of adsorption and desorption of individual proteins. We are also interested in the competitive nature of these processes, e.g., the adsorption of two or more proteins simultaneously or the replacement of an adsorbed protein by another.

3) Determination of the effect of adsorption on the reaction of enzyme-protein systems. This includes studies of the effects of adsorption on the enzyme, the substrates, and on any end product.

Three distinct techniques have been employed in this investigation and are described separately below in some detail.

Ellipsometry

Ellipsometry is quite unique in its ability to give a measure of the average dimensions of an undisturbed protein layer adsorbed on a surface. Briefly, ellipsometry is an optical technique in which the change in state of polarization of light upon reflection from a surface is used to characterize the surface and to determine the thickness and refractive index of a thin film overlaying that surface. The thickness and refractive index of this film can be determined simultaneously from these measurements. From these independently determined quantities, one can also determine the concentration of protein in an adsorbed film as well as the amount of protein adsorbed on a surface. The technique has the advantage that measurements can be made *in situ*, that is, during the time required for interactions and changes in molecular shape and without introducing any artifacts due to removal of the surface from the solution.

A wide variety of sample types, both metallic and dielectric, may be studied. The only general requirements are that the surface or interface be well defined and smooth enough to give specular reflection and that it be stable under the experimental conditions. The sensitivity of the measurement is dependent upon the details of the experimental system, mainly the refractive indices of the solution, film, and surface involved; films as thin as a few angstrom units can be measured under the proper conditions.

A typical system for study by the ellipsometer consists of a film of index n_2 and thickness d on a reflecting substrate of index n_3 immersed in a medium of index n_1, as shown in Figure 1. Let all media be isotropic and n_1 represent a real index of refraction, while n_2 and n_3 may be complex.

Consider light incident at the boundary between the immersion medium and film. The cosine of the refraction angle is

$$\cos\phi_2 = \left[1 - \left(\frac{n_1}{n_2} \sin\phi_1 \right)^2 \right]^{1/2} \qquad (1)$$

The parallel and normal reflection coefficients for light incident at this boundary are:

$$r_{12}^p = \frac{n_2\cos\phi_1 - n_1\cos\phi_2}{n_2\cos\phi_1 + n_1\cos\phi_2} \qquad (2)$$

and

$$r_{12}^s = \frac{n_1\cos\phi_1 - n_2\cos\phi_2}{n_1\cos\phi_1 + n_2\cos\phi_2} \qquad (3)$$

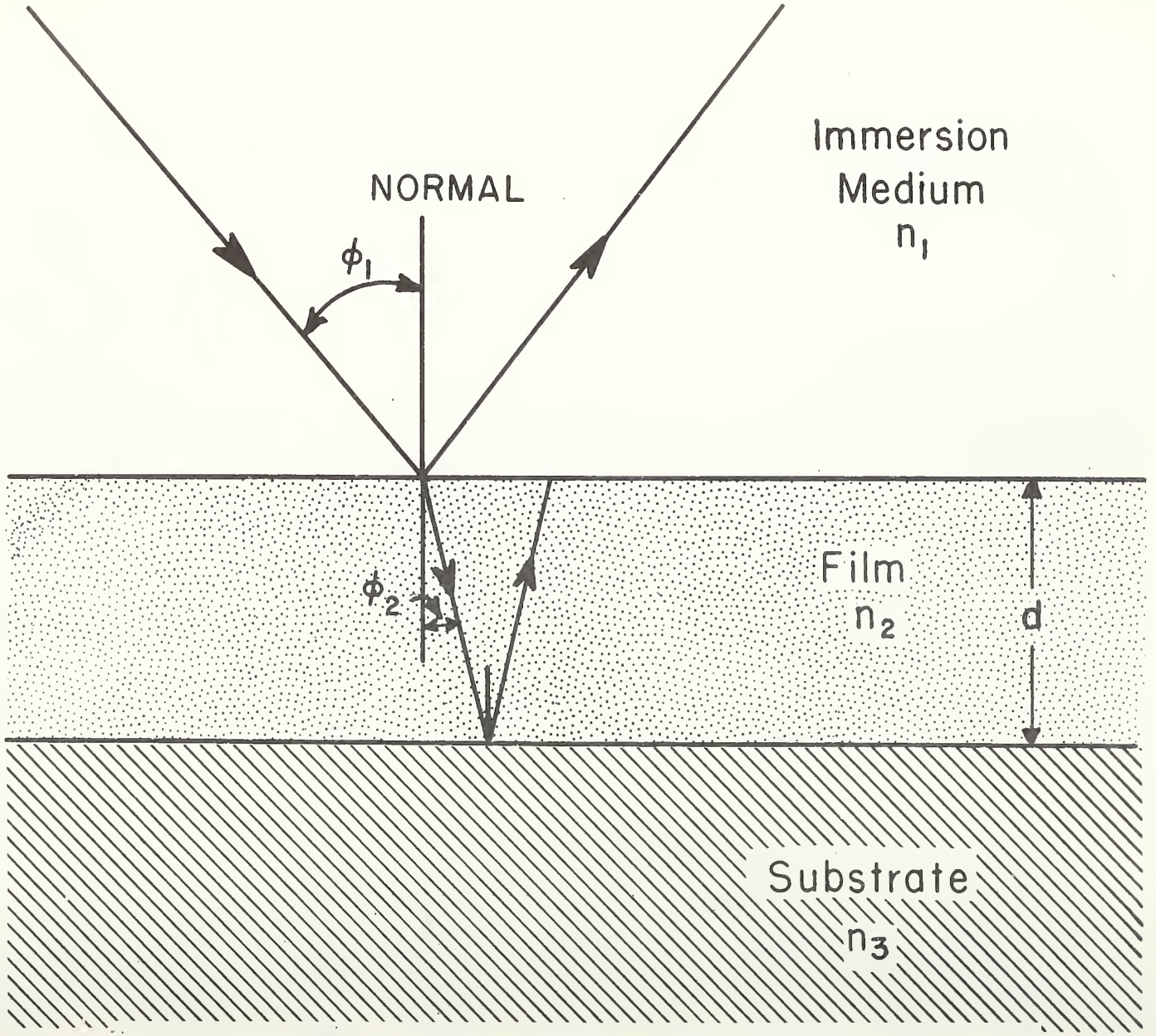

Figure 1: Schematic representation of reflection from film covered surfaces

respectively. The reflection coefficients, r_{23}^p and r_{23}^s at the boundary between the film and substrate are given by similar expressions.

The total reflection coefficients, R^p and R^s that include the contributions of reflections from lower boundaries are given (15) by:

$$R^p = \frac{r_{12}^p + r_{23}^p \exp D}{1 + r_{12}^p r_{23}^p \exp D} \tag{4}$$

and

$$R^s = \frac{r_{12}^s + r_{23}^s \exp D}{1 + r_{12}^s r_{23}^s \exp D} \tag{5}$$

where $\cos \phi_3$ values needed for these reflection coefficients are given by an expression similar to eq (1) and D represents the quantity

$$D = -4\pi i n_2 \cos\phi_2 d_2/\lambda \tag{6}$$

where λ is the wavelength of the light used, in vacuum, and $i = \sqrt{-1}$. The ratio of the parallel and normal total reflection coefficients is defined as ρ:

$$\rho = R^p/R^s. \tag{7}$$

This may be expressed in terms of the relative attenuation and phase shift of the parallel component with respect to the perpendicular component that occurs, represented by the azimuthal angle Δ, and relative phase shift ψ by:

$$\rho = \tan\psi \exp(i\Delta). \tag{8}$$

Thus ρ is determined from ellipsometer readings.

The value of the complex index of a reflecting surface can be calculated from the equation

$$n_3 = n_1 \tan\phi_1 \left[1 - \frac{4\rho \sin^2 \phi_1}{(\rho+1)^2} \right]^{1/2} \tag{9}$$

where ϕ_1 is the angle of incidence and ρ is determined from ellipsometry measurements on the base substrate.

Further details of the technique are given in references 16-20 and our application to the very analogous problem of the adsorption of synthetic polymers from solution is given in references 21-25. In summary, for a bare metallic surface two parameters are obtained from the instrument readings: tan ψ, the ratio of the magnitude of the reflection coefficient for light polarized with its electric vector in the plane of incidence to that polarized normal to the plan of incidence, and Δ, the relative phase difference for these two polarizations. From these measured quantities it is possible to calculate directly the optical constants of the surface. All other parameters required for this calculation such as the wave length of light, refractive index of immersion medium, etc., are obtained by independent means. If this surface is now covered with a thin film, different values of ψ and Δ result. From these new values and the previously determined values of the optical constants of the surface, the thickness and refractive index of the film can be calculated by the equations of Drude (15). Although these equation are not solvable in closed form, answers may be determined to any desired degree of accuracy by iterative methods, necessitating the use of electronic computers for most applications (26).

Shown in Figure 2 is a schematic diagram of the ellipsometer. Photographs of the instrument are given in Figures 3 and 4. In normal use, the cell is surrounded by a constant temperature jacket. A diagram of the cell is shown in Figure 5.

The model treated in the Drude equation is that of a homogeneous film of constant refractive index with discrete boundaries. However, an adsorbed polymer film, consisting of a mixture of polymer and solvent, would be expected to be an inhomogeneous film, with the polymer concentration and hence refractive index decreasing with the distance from the surface. The use of the Drude equations will result, therefore, in an "average" thickness value and it is necessary to relate this calculated average from a homogeneous film model to the inhomogeneous adsorbed polymer film (27), and if possible, to molecular parameters.

Infrared Difference Spectroscopy

The interaction of the chromophores of an adsorbed molecule with a surface frequently results in a shift of their characteristic spectral adsorption bands. Typically, for the protein studies presented here, a shift of -20 cm^{-1} of the amide I band for free and bound carbonyl groups was observed. (See Figure 6.) If these bands were well resolved, one could immediately use the optical density of the shifted band to determine the bound fraction, p, the fraction of the carbonyl chromophores of an adsorbed protein molecule directly in contact with the surface. Knowledge of the amino acid composition of the protein would then enable a calculation of the average number of carbonyl attachments per molecule.

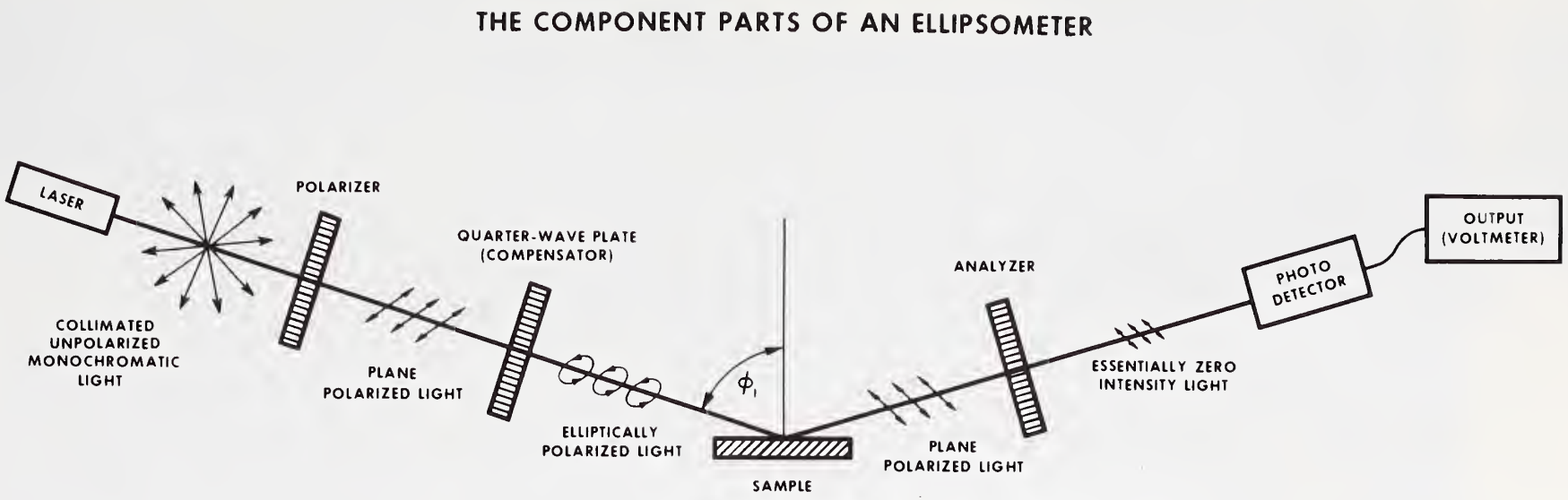

Figure 2: Schematic representation of ellipsometer.

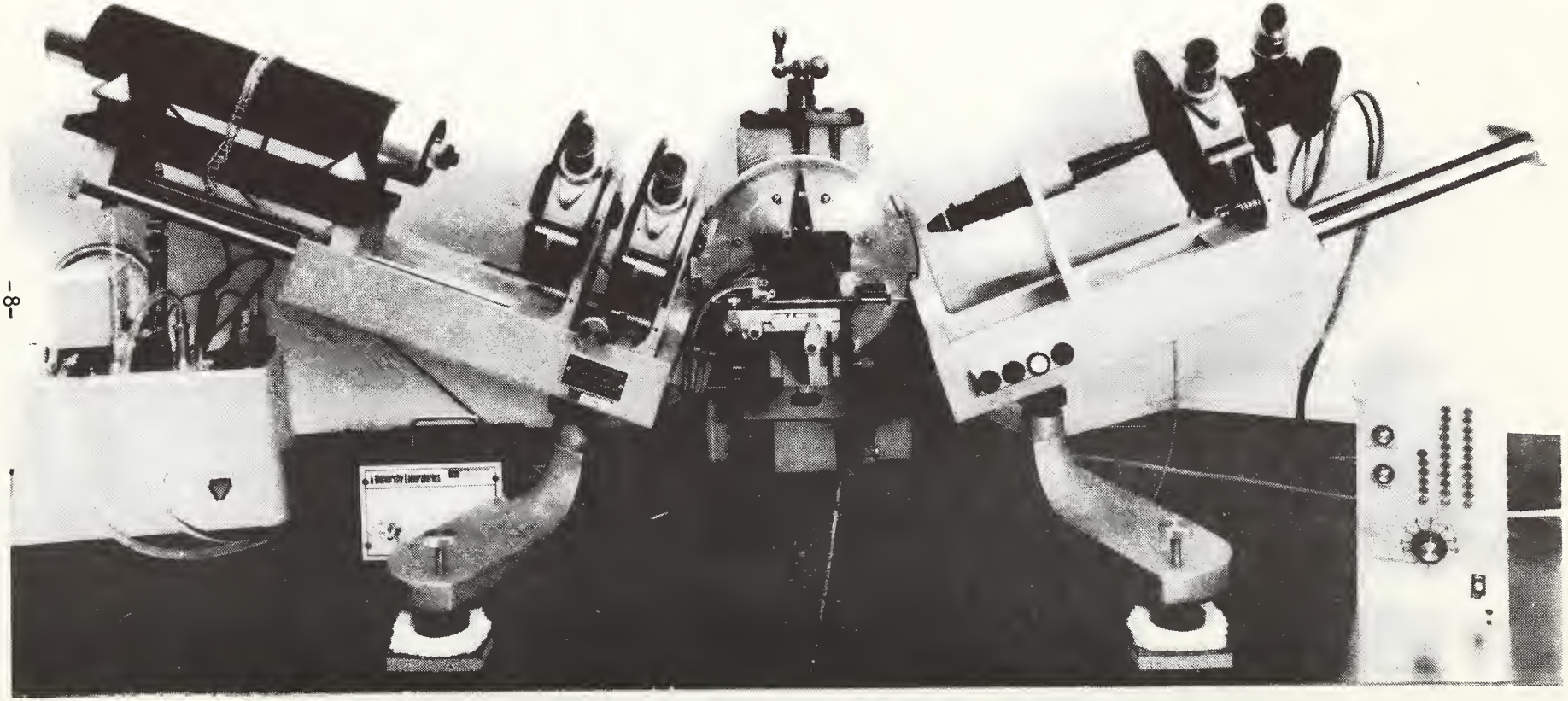

Figure 3: Photograph of ellipsometer

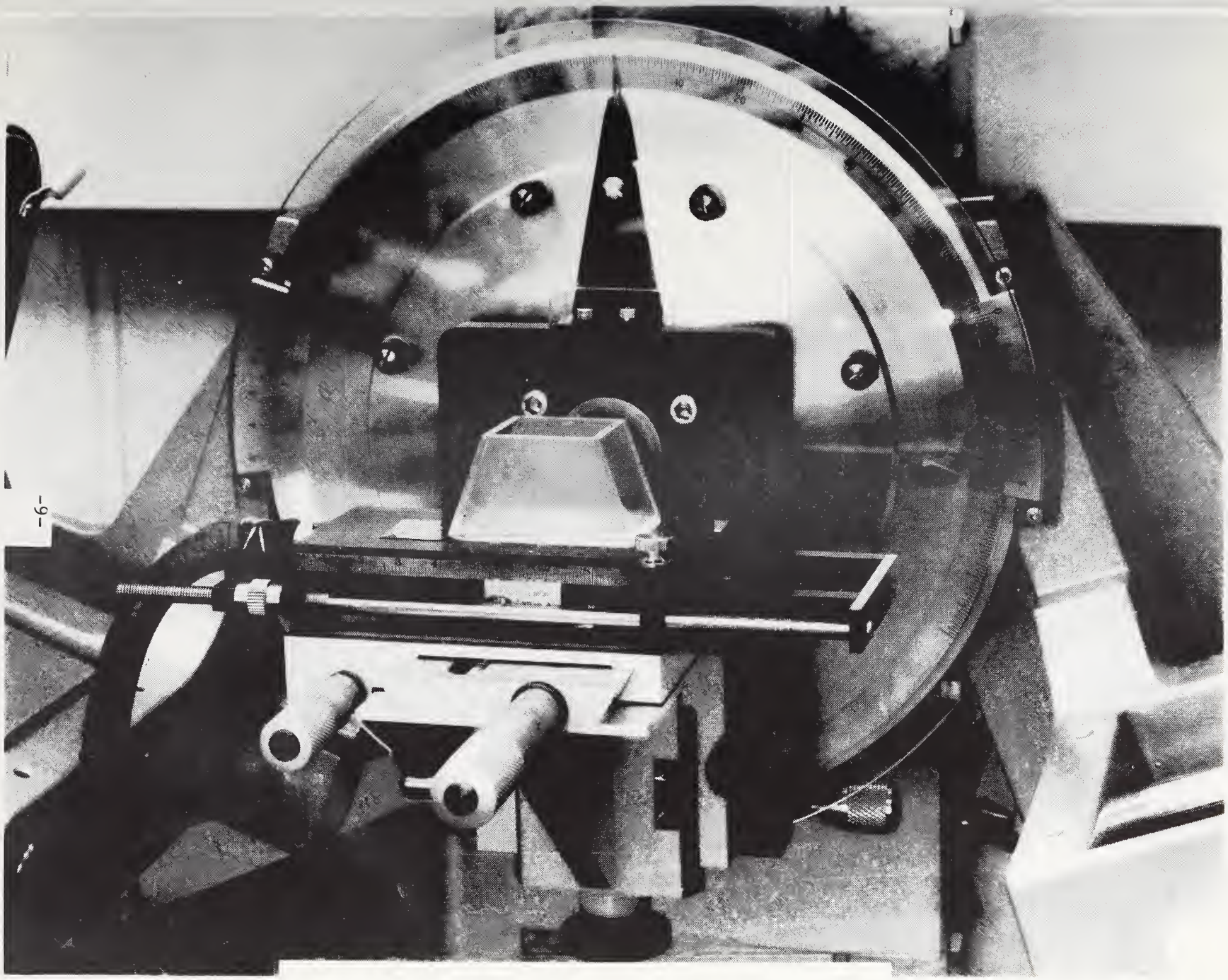

Figure 4: Photograph of ellipsometer stage with cell

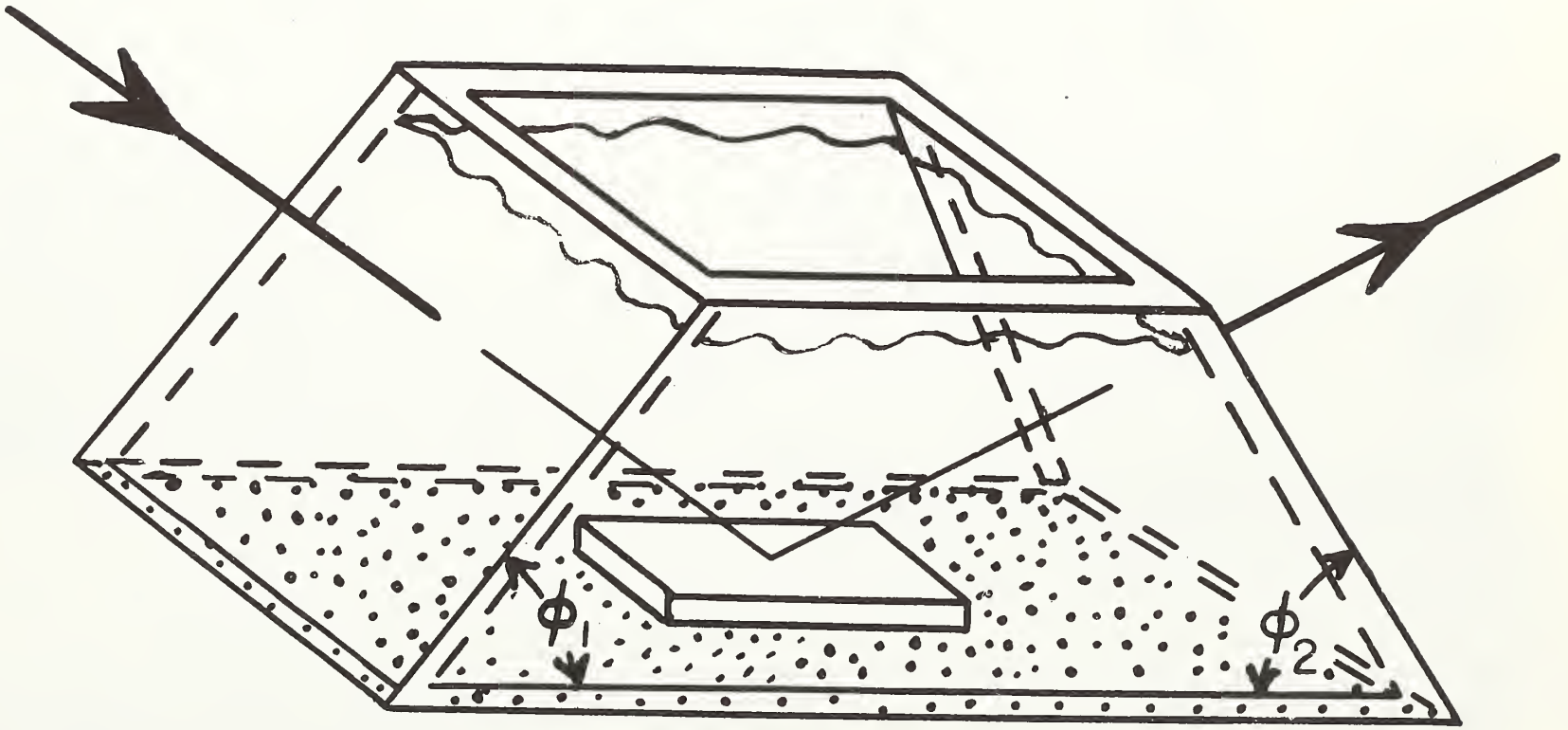

Figure 5: Schematic diagram of adsorption cell

I. R. SPECTRAL SHIFT

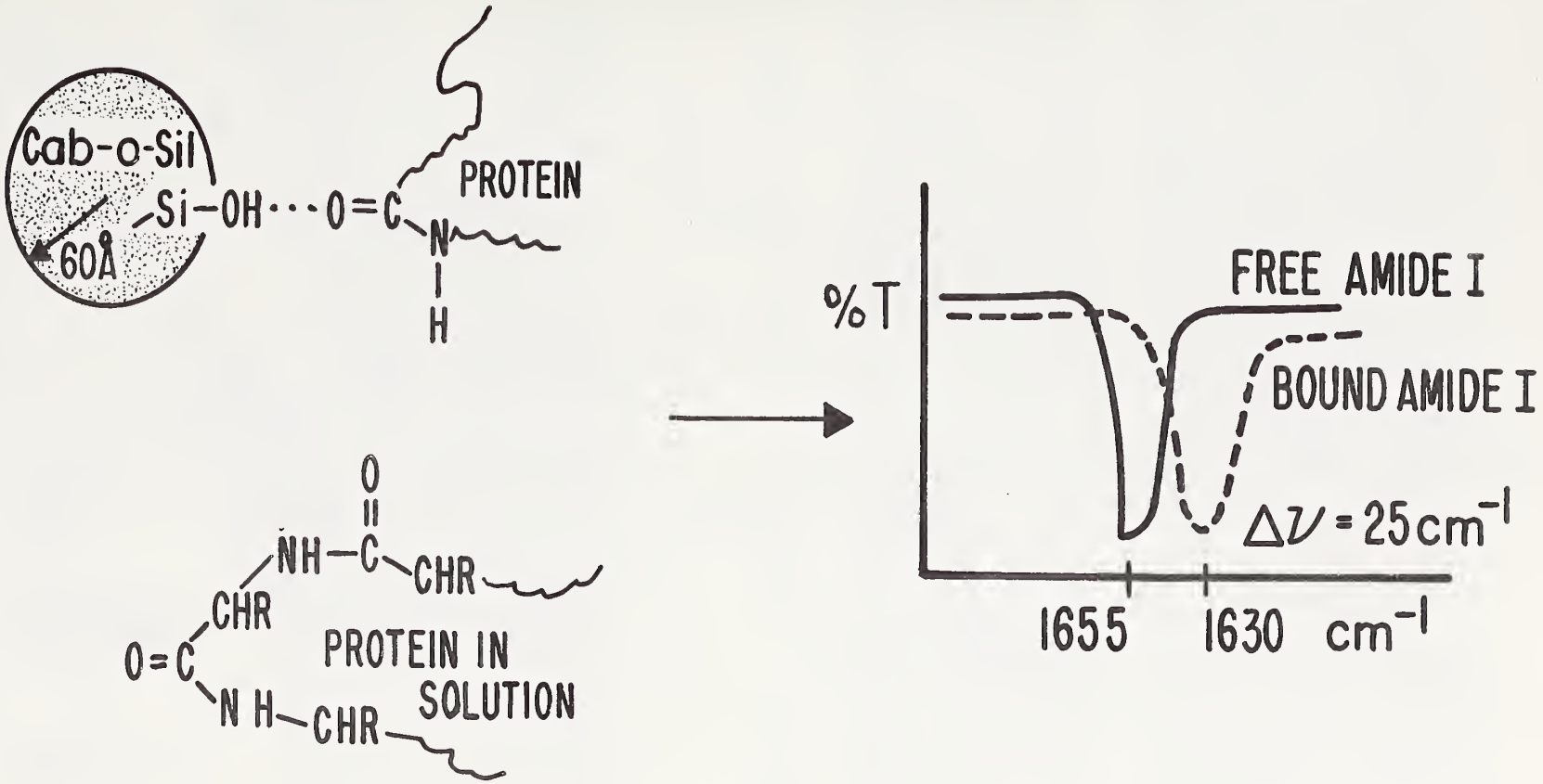

Figure 6: Shift in absorption frequency of amide I band as a result of carbonyl group interaction with surface.

Usually, there is a significant overlap of the two bands and difference techniques must be used. This is illustrated in Figure 7. Fontana and Thomas (28) originally described a method whereby a polymer is adsorbed from solution on a high surface-area powder, the suspension centrifuged, and an infrared difference spectrum recorded for the resulting gel. The signal due to the solvent and unbound chromophores of the adsorbed polymer are compensated by adjusting the pathlength of the reference cell containing polymer solution. This results in a resolved spectrum of the adsorbed chromophores. This technique was modified (29) to permit a direct analysis of the suspension, thereby removing uncertainties in polymer concentration and has been used by a number of investigators (30-32) to study the conformation of synthetic polymers adsorbed from organic solvents.

For the determination of the bound fraction for an adsorbed protein, it is necessary to use D_2O solutions of deuterated proteins to obtain a window in the 1650 cm^{-1} region. Although the shoulder of a D_2O band exists just below this region, preventing the use of a variable pathlength reference cell, matched cells can be employed, and the following analysis was used to resolve the difference spectrum arising from a protein solution-silica suspension in the sample beam versus the same protein solution in the reference beam (see Figure 7). A difference spectrum for bovine serum albumin is shown in Figure 8.

The absorbance at the frequency ν^o of the unshifted band is given by Beer's law

$$A^o = \varepsilon^o C_f L + \varepsilon^o C_{u,a} L + \varepsilon_a^o C_b L - \varepsilon^o C_r L_r \qquad (10)$$

where ε^o and ε_a^o are the extinction coefficients of the unbound and bound carbonyl groups at the frequency ν^o, L and L_r are the path lengths of the sample and reference cells, C_f is the concentration of unbound groups of free protein in the suspension, $C_{u,a}$ is the concentration of unbound groups of adsorbed protein, C_b is the concentration of bound groups, and C_r is the concentration of carbonyl groups in the reference cell. With the total concentration of groups $C = C_f + C_b + C_{u,a}$, and realizing that $\varepsilon^o C_r L_r = \varepsilon L(C + C_d)$, where C_d is the change in concentration (dilution effect) due to the addition of the non-porous powder, the bound fraction, p, which is defined as C_b/C_a where C_a is the concentration of all groups on adsorbed molecules (i.e., change in protein solution concentration as a result of adsorption), is given by

$$p = \frac{A^o + \varepsilon^o C_d L}{C_a L(\varepsilon_a^o - \varepsilon^o)_{\nu^o}} . \qquad (11)$$

I. R. DIFFERENCE MEASUREMENTS

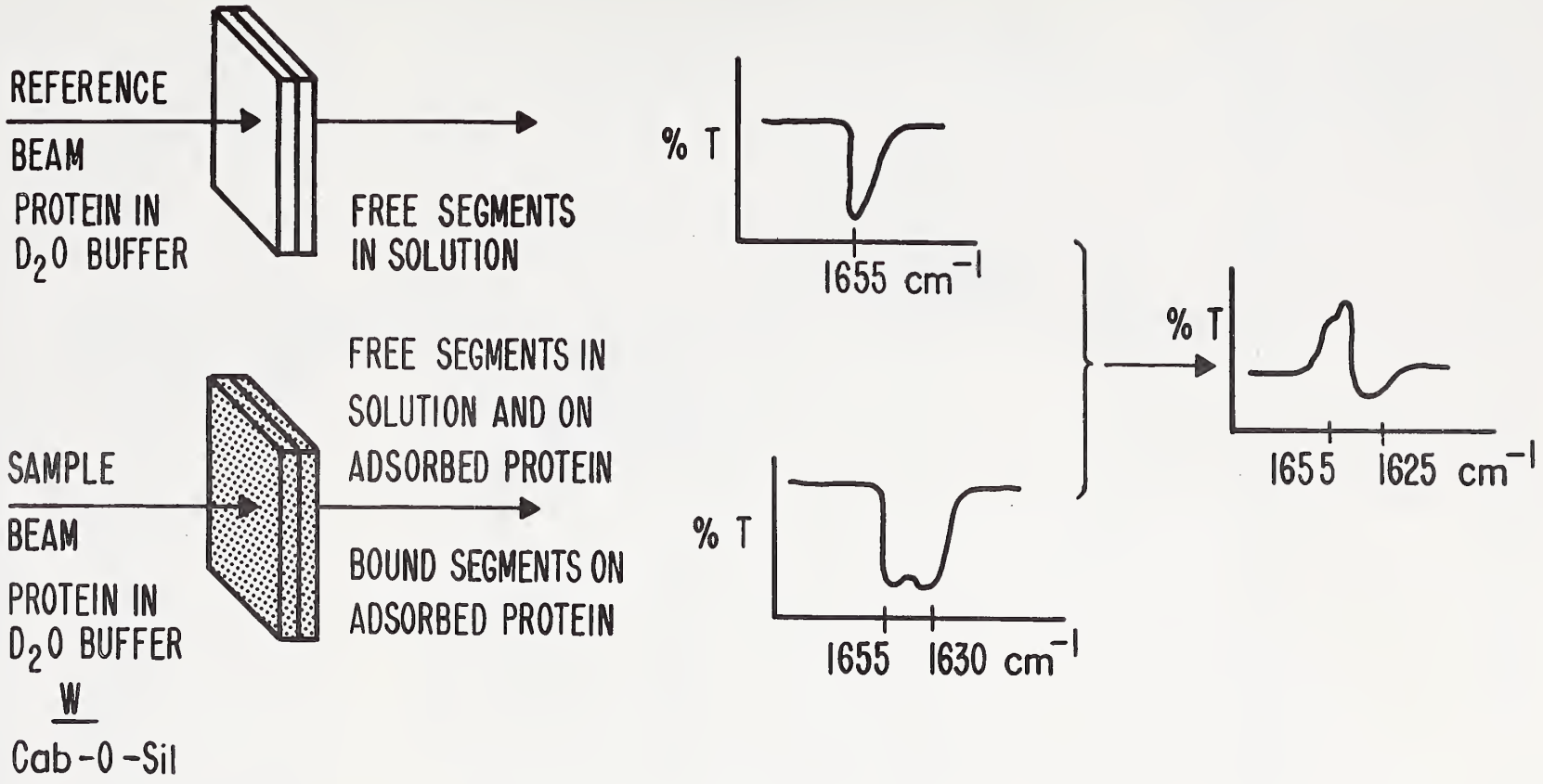

Figure 7: Experimental method for infrared difference measurements

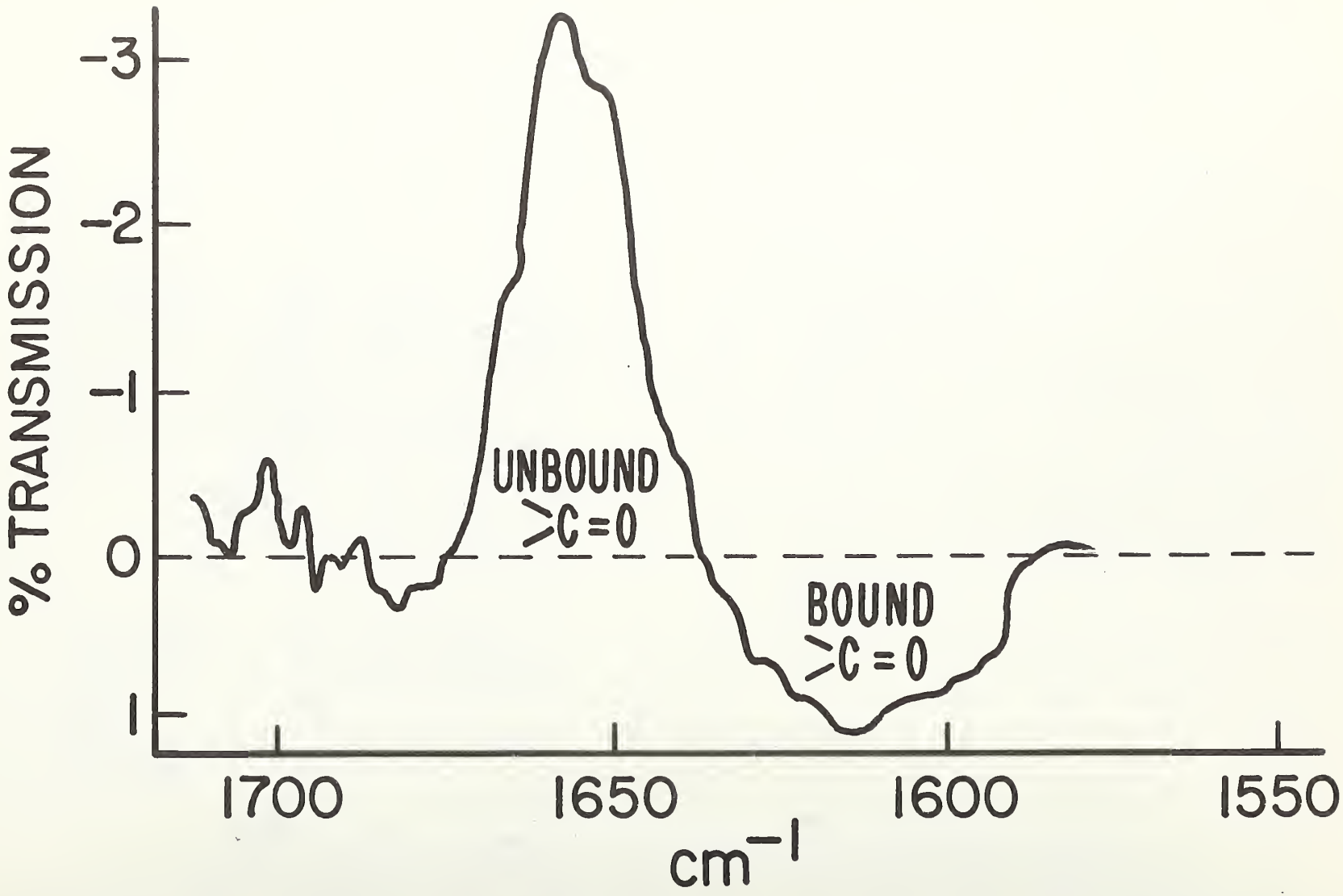

Figure 8: Infrared difference spectrum of bovine serum albumin adsorbed on silica.

To determine ε_a^o, the reference solution is diluted until the signal due to unbound groups is nulled. This establishes the condition $\varepsilon^o C'_r L_r = \varepsilon^o L(C_f + C_{u,a})$, which in conjunction with Eq. 10, yields

$$C_b = \frac{A^{o\prime}}{\varepsilon_a^o L} , \tag{12}$$

where $A^{o\prime}$ is the new absorbance at ν^o. Elimination of C_b between Eqs. 10 and Eq. 12, expressing the result in terms of known quantities, and solving for ε_a^o, yields

$$\varepsilon_a^o = \frac{A^{o\prime}\varepsilon^o}{A^{o\prime} - A^o - \varepsilon^o L C_d} . \tag{13}$$

Computer Simulation

The computer simulation studies of the adsorption of lysozyme utilized the known atomic coordinates (33) determined from X-ray crystallographic studies. For convenience in these calculations, a coordinate system with the X axis passing through the amino acid residues glycine 126 and proline 70, and Y axis passing through glycine 126 and alanine 42, was chosen, as shown in Figure 9. The process of adsorption of a given orientation of lysozyme onto a planar surface was simulated as follows. For a given orientation of the lysozyme molecule with respect to the surface, the molecule was moved toward the surface until the first contact was established between an atom of the molecule and the surface. The molecule and surface were then held fixed. The question was then asked: At what distance normal to the surface should a hypothetical plane parallel to the surface be placed such that a given number of carbonyl groups are located between the plane and the surface? This distance was then determined, the molecule removed from the surface, rotated, and the process repeated. All possible orientations of lysozyme with respect to the surface resulting from rotation in 5° increments about the X and Y axes were tested. The vicinity of the minimum distance determined by this procedure was then probed in 1° increments.

Radiotracer

As the adsorption of proteins from plasma occurs from a complex mixture, their rates of competitive adsorption are fundamental in determining the eventual state of any given protein. Such rates must be accurately determined on the single proteins before the effects of competitive adsorption can be assessed. Radiotracers are convenient for the accurate measurement of amounts of adsorbed protein. The adsorbing solution concentrations can be varied over a wide range and adsorption times on the order of 5-10 seconds can be studied. Furthermore, by the proper selection of tagged components, the relative amounts adsorbed of two or more proteins can be determined even when adsorbed simultaneously from the same solution. We have previously applied radiotracer techniques to studies of the adsorption of labeled synthetic polymers utilizing both tritium and ^{14}C.

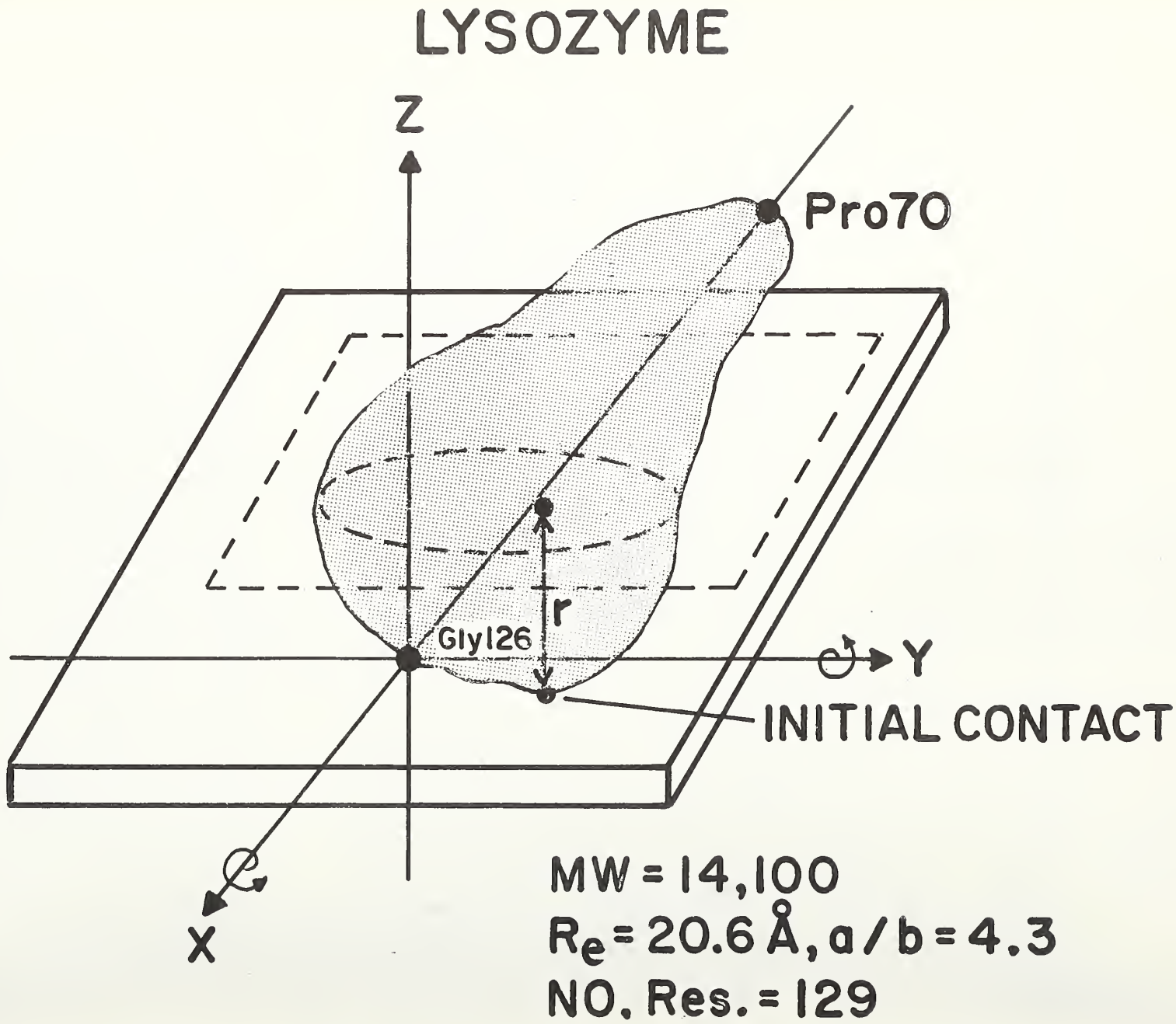

Figure 9: Schematic representation of lysozyme adsorbed on planar surface showing coordinate system used for computer calculation.

A major problem in the investigation of protein adsorption by radiotracer techniques is the removal of the sample from the adsorption solution without transferring any of the protein from the air-solution interface. It is also necessary to wash away the "carry-out" solution without desorbing any of the adsorbed protein. However, a technique that includes washing, no matter how short the washing period, may remove an unknown amount of adsorbed polymer. Comparison of the adsorbed quantities with those obtained by other techniques, such as ellipsometry, however, permits an evaluation of the quantities that we removed by a washing process.

EXPERIMENTAL

Ellipsometry

The following materials were used for the ellipsometric studies. Human serum albumin (4x crystallized), bovine prothrombin (Cohn III-2), and ε-aminocaproic acid were obtained from Nutritional Biochemicals (NBC)* and used without further treatment. Human fibrinogen (NBC) was purified by the procedure of Batt, et.al. (34), a modification of a procedure due to Laki (35).

Commercial ferrotype plate consisted of chromium electroplated on either brass or stainless steel. Platinum was of 99.95% purity and rolled to a mirror finish by Engelhard Industries. Quartz was fused silica, no. 7940, from Corning Glass. These materials were cleaned before use with hot 50:50 HNO_3:H_2SO_4 followed by three rinses in boiling distilled water. They were then heated for five minutes at 500° in a muffle furnace and placed while still warm into the adsorption cell filled with buffer.

Low temperature laminar (LTL) type pyrolytic graphite was kindly supplied by Dr. Jack Bokros of General Atomic Corp. Commercial polyethylene sheet was cleaned in ethanol and cast under vacuum at 180° between plate glass sheets. Several samples were prepared from NBS Standard Reference Material 1475 linear polyethylene which has a density of 0.978 g/cm^3, also by casting against glass under vacuum. All results on these two types of polyethylene were identical. The carbon and polyethylene samples were cleaned before use by washing with ethanol for two weeks in a Soxhlet extractor followed by a similar washing with distilled water.

All solutions were made up in pH 7.4 phosphate buffer and concentrations were determined by UV spectroscopy. All adsorption measurements were made in a cell thermostatted at 37°C.

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*Certain commercial materials and instruments are identified here and elsewhere in this publication in order to adequately specify the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the equipment or instruments identified are necessarily the best available for the purpose.

Each adsorption run consisted of first determining the optical constants of the bare surface while under the buffer solvent. Buffer was then withdrawn with a syringe and the cell refilled with additional buffer and the optical constants redetermined. Solvent exchange was repeated until no change in optical constants was observed. If changes were observed after three such exchanges, the surface was discarded. The solvent was then exchanged for solution and measurements were taken as a function of adsorption time. Due to refractive index inhomogeneities in the adsorption cell, stable readings cannot generally be made in less than ten minutes following any exchange of solvent or solution. Blank runs made with surfaces kept under buffer for several days established the amount of drift in ellipsometer readings to be expected for these time periods. In general, drift was negligible ($\leq 0.05°$) for times up to 24 hours. Longer times occasionally led to substantial changes.

Infrared Difference Spectrometry

Bovine serum albumin (4x crystallized), bovine fibrinogen (Cohen fraction I, 60% clottable), bovine prothrombin (fraction III-2), and lysozyme (chicken egg white, 3x crystallized) were obtained from Nutritional Biochemicals. The serum albumin and lysozyme were deuterated (36), lyophilized, and stored in vacuo at 4°C. The prothrombin was dissolved in 0.1M D_2O phosphate buffer pD 7.4, dialyzed overnight against this buffer, and filtered through a well washed 0.8 μm pore size filter just prior to use. Fibrinogen was purified by the method of Laki (35) with the following modifications: The ammonium sulfate precipitate was resuspended in the pD 7.4 phosphate buffer and dialyzed overnight aginst the same buffer. The resulting solution was filtered and used immediately.

Poly-L-lysine and poly-L-glutamic acid of average molecular weights 139,000 and 102,700, respectively, were obtained from Nutritional Biochemicals. The polyamino acids were deuterated prior to use by dialyzing overnight against D_2O-phosphate buffer. The α-helix and random coil forms of poly-L-lysine were obtained by adjusting (37) a portion of the deuterated material to pD 11.5 and 4.8 with Dcl and NaOD, while the helix and coil forms of poly-L-glutamic acid were generated by adjusting (38) the pD to 5.4 and 11.4. Polyamino acid concentration changes were measured spectrophotometrically (39) using the optical density difference at 215 and 225 nm.

The mild heat denaturation of serum albumin was carried out at 66°C (40). The quantitative, limited, reduction of disulfide bonds of serum albumin was prepared by the method of Sela et. al. (41) with the omission of urea and a reaction time of 1.5 hours.

The cross-linking of serum albumin was accomplished using diethyl malonimidate dihydrochloride prepared from malononitrile and HCl gas in anhydrous ethanol-dioxane according to McElvain and Schroeder (42). The reaction with serum albumin generally followed Dutton et. al. (43), using a 1.5% protein solution with reaction for 1 hour.

The 0.1M D_2O phosphate buffer was prepared using 99.8% D_2O and deuterated salts. Measurements of pD were standardized against NBS standard reference materials 2186-I and 2186-II. The pD of solutions was adjusted with concentrated DCl and NaOD. Ionic strength adjustments were made with KCl.

The adsorbent used for all experiments was a fumed, non-porous silica with a nominal particle size of 0.012 μm (Cab-O-Sil M-5, Cabot Corp.) The adsorbent was heated in vacuo at 110°C overnight and stored over silica gel just prior to use. The surface area, as determined by BET N_2 analysis, was found to be 204 ± 20 m^2/gm.

Data for adsorption isotherms were obtained at 23.5 ± 0.5°C by shaking known amounts of silica and protein solution for three hours (judged to be equilibrium from rate studies showing no further increase in adsorbance). The silica was then removed by filtration. The amount of adsorbed protein was determined from the protein concentration changes measured spectrophotometrically at 280 nm using accepted extinction coefficients for fibrinogen and prothrombin (44), serum albumin (45), and lysozyme (46). To check for contamination, the ratio of silica to solution volume was varied with no effect on the protein adsorption isotherms. For studies of adsorbance as a function of time, the quantities of silica and protein solution were suitably scaled up and at various times aliquots withdrawn and filtered.

For bound fraction studies, 0.029gm silica per ml protein solution was found generally to give stable suspensions suitable for infrared analysis. The difference spectrum of the protein-silica suspension versus the same protein solution was obtained from the region 1750-1550 cm^{-1} using matched 0.1 mm CaF_2 cells at 10x ordinate expansion. The extinction coefficient, ε_a°, was obtained by scanning successive dilutions of the reference solution against the protein-silica suspension and noting the dilution at which the peak due to the unbound chromophores diappeared. Each reported bound fraction value represents the average of from two to four infrared scans on the same protein suspension.

While contact of the D_2O solutions with the atmosphere can lead to the incorporation of H_2O, the rapid conversion $H_2O + D_2O \rightarrow 2HOD$ insured a window in the 1650 cm^{-1} region. The resulting HOD, however, does obscure the 1500 cm^{-1} region where the amide II band involving N-H occurs. Therefore, only contacts of the carbonyl group were measured and used to calculate the bound fractions reported in this paper.

Radiotracer

The radiotracer experiments utilized the same chrome and platinum as used for the ellipsometry studies. ^{131}I labeled human albumin was obtained from Squibb and quartz from General Electric (Type 151). The chrome slides were cleaned in hot benzene followed by 10 minutes of heating in a muffle furnace at 500°C. The quartz and platinum were cleaned with hot 1:2 HNO_3:H_2SO_4 for one hour followed by heat treatment at 500°C

for 10 minutes. The slides were placed in buffer while still hot and were never subsequently exposed to air.

^{14}C hexadecane and ^{14}C stearic acid used for surface roughness determinations were obtained from New England Nuclear Corp.

Solutions of the unlabeled protein were made up by weight in pH 7.4 phosphate buffer and ^{131}I labeled protein solution was added by syringe. Adsorption was carried out in a covered glass vessel equipped with a magnetic stirrer and wrapped with heating coils to maintain a temperature of 37°C.

In order to establish the specific activity of the labeled protein solutions, 1 to 10 λ aliquots of the solution were taken and evaporated to dryness on steel planchets. These were counted at the same time as the adsorption samples to minimize decay corrections. Measured activities could be made independent of sample size by coincidence time corrections but these were usually high for the large aliquots. Separate experiments established that the quartz and chrome samples had the same backscatter as the steel while the platinum showed 25% more backscatter and the activities were adjusted for this.

All counting was done in a Nuclear Chicago 1105 low background beta counter with a mylar window operating in the G-M mode. The adsorption slides were always masked with a lead sheet containing a circular hole in order to maintain consistent counting geometry, define the counted area, and to eliminate edge effects.

MOLECULAR EXTENSION

Time Dependent Behavior

Two aspects of the ellipsometric results should be considered: a) the time dependence of the adsorption process and b) the long time or equilibrium values. For example, Figures 10 and 11 show the time dependence of the thickness or molecular extension and adsorbance, respectively, of fibrinogen adsorbed on chromium. The error bars in Figure 10 are calculated maximum uncertainties based upon estimates of instrumental precision. The corresponding error limits in Figure 11 are smaller than the sizes of the points. While no change is seen in the extension during the time period shown, there is a small but definite increase in adsorbance. Rates should not be inferred from these results as the ellipsometer cell was not stirred for these experiments. Although duplicate runs, in general, may give somewhat different values of extension and adsorbance, the general features of increasing adsorbance with constant extension are always observed within an individual set of data for this protein and surface. Extension measurements made at adsorption times shorter than 20 minutes generally show greater scatter, but they are generally randomly displaced from the average value.

The constant value of extension with time can be interpreted as a lack of significant molecular rearrangement of the protein molecule once adsorption has taken place. In the early time periods, additional molecules are still arriving at the surface as evidenced by the increasing adsorbance, but the surface pressure of these molecules is not sufficient to cause the molecules already adsorbed to change their conformation. Nor, apparently, do the late arrivals adopt a significantly more extended conformation than the majority of the adsorbed molecules. It is recognized that conformational changes may occur in the first few seconds or minutes of adsorption. However, it should be noted that a random coil homopolymer has been shown (22) to undergo a significant change in extension during the time periods accessible by this technique, indicating more conformational freedom than that shown by the proteins studied here.

The absence of change in extension with adsorption time is a general feature found for all three proteins on all of the surfaces examined to date. Further examples can be seen in the data for the adsorption of prothrombin on chromium (Figures 12 and 13), laminar carbon (Figures 14 and 15), and polyethylene (Figures 16 and 17). The differences in the error limits of adsorbance seen in these figures is due to the low refractive index of polyethylene causing a loss of sensitivity.

An increase in adsorbance was not always observed. An example of this is shown in Figure 18 which gives the adsorbance of albumin on chrome where no discernable trend can be seen. Under certain conditions, a decrease in adsorbance with time has been noted when unpurified Cohn Fraction I fibrinogen was adsorbed. The adsorption of Cohn Fraction I on the high surface energy metals and quartz was qualitatively the same as the purified

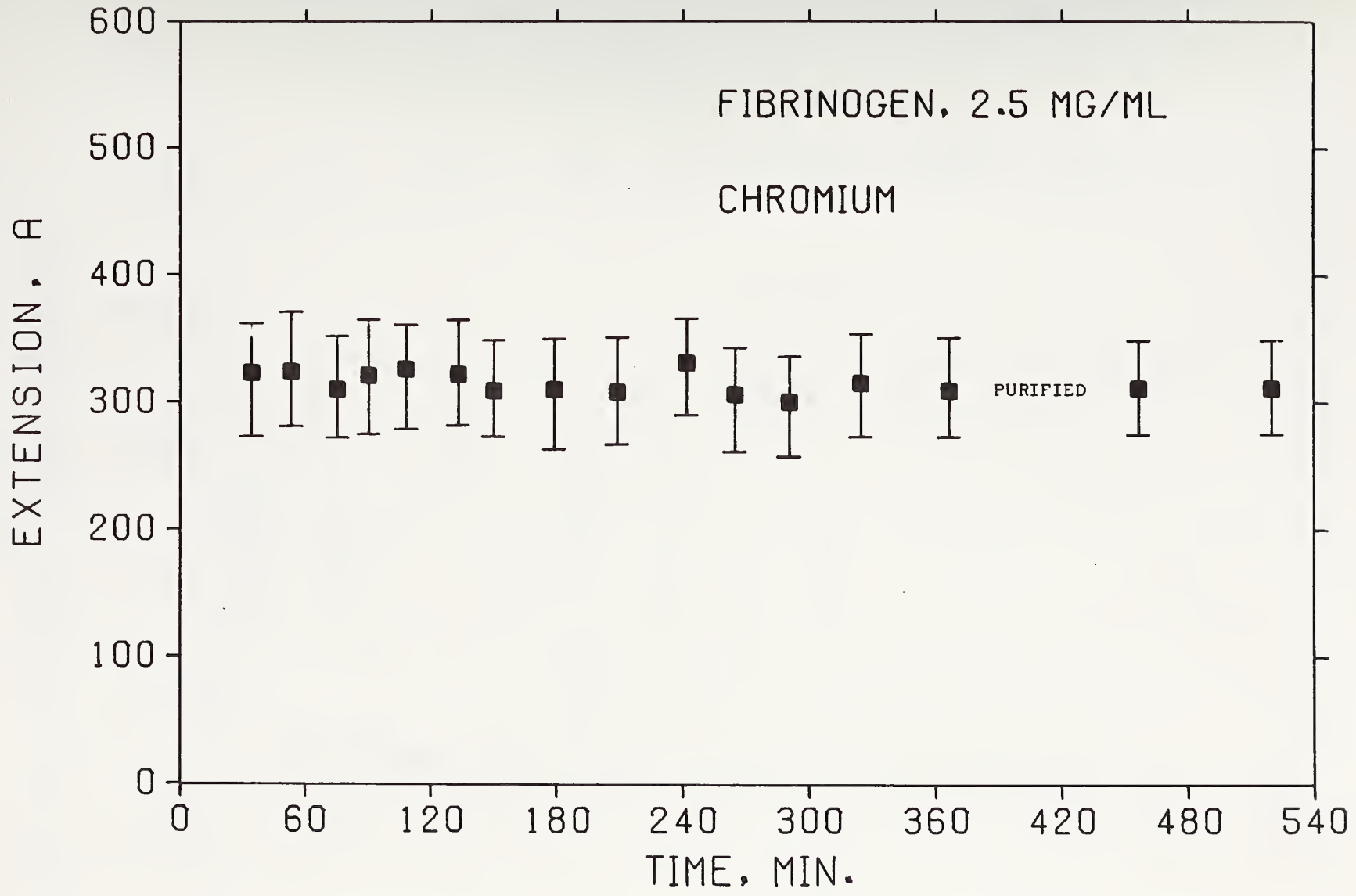

Figure 10: Extension of fibrinogen on chromium

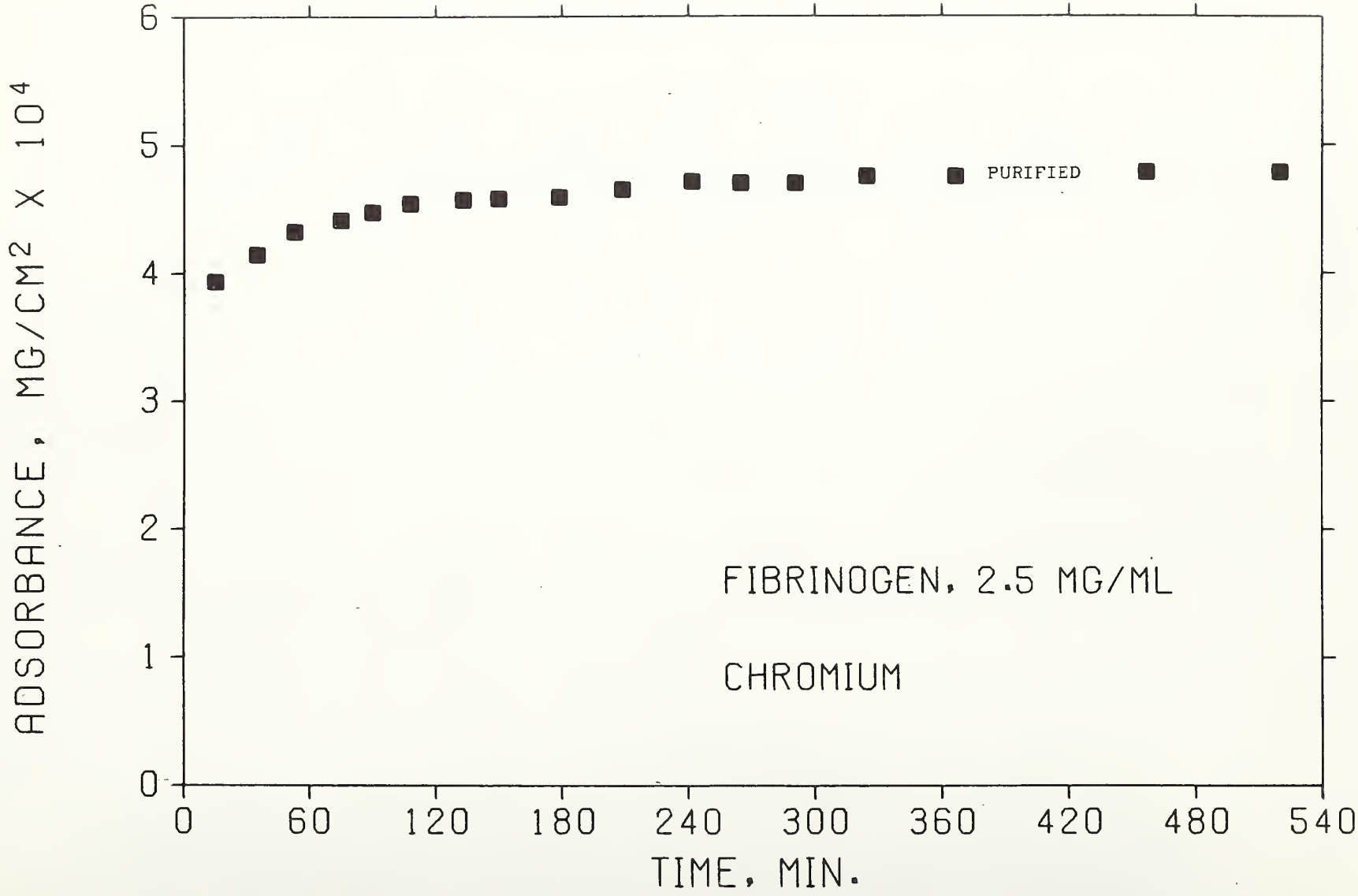

Figure 11: Adsorbance of fibrinogen on chromium

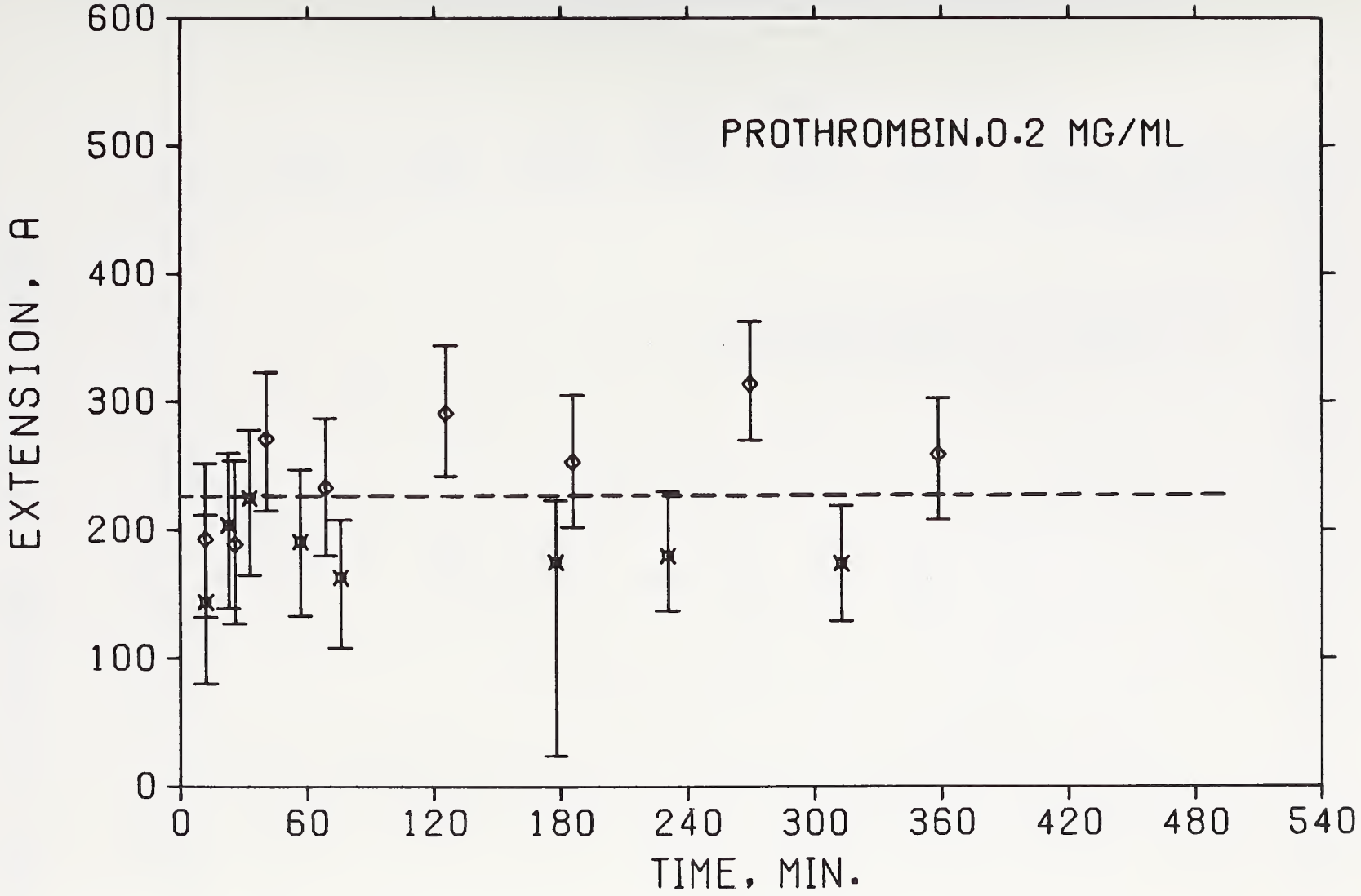

Figure 12: Extension of prothrombin on chromium

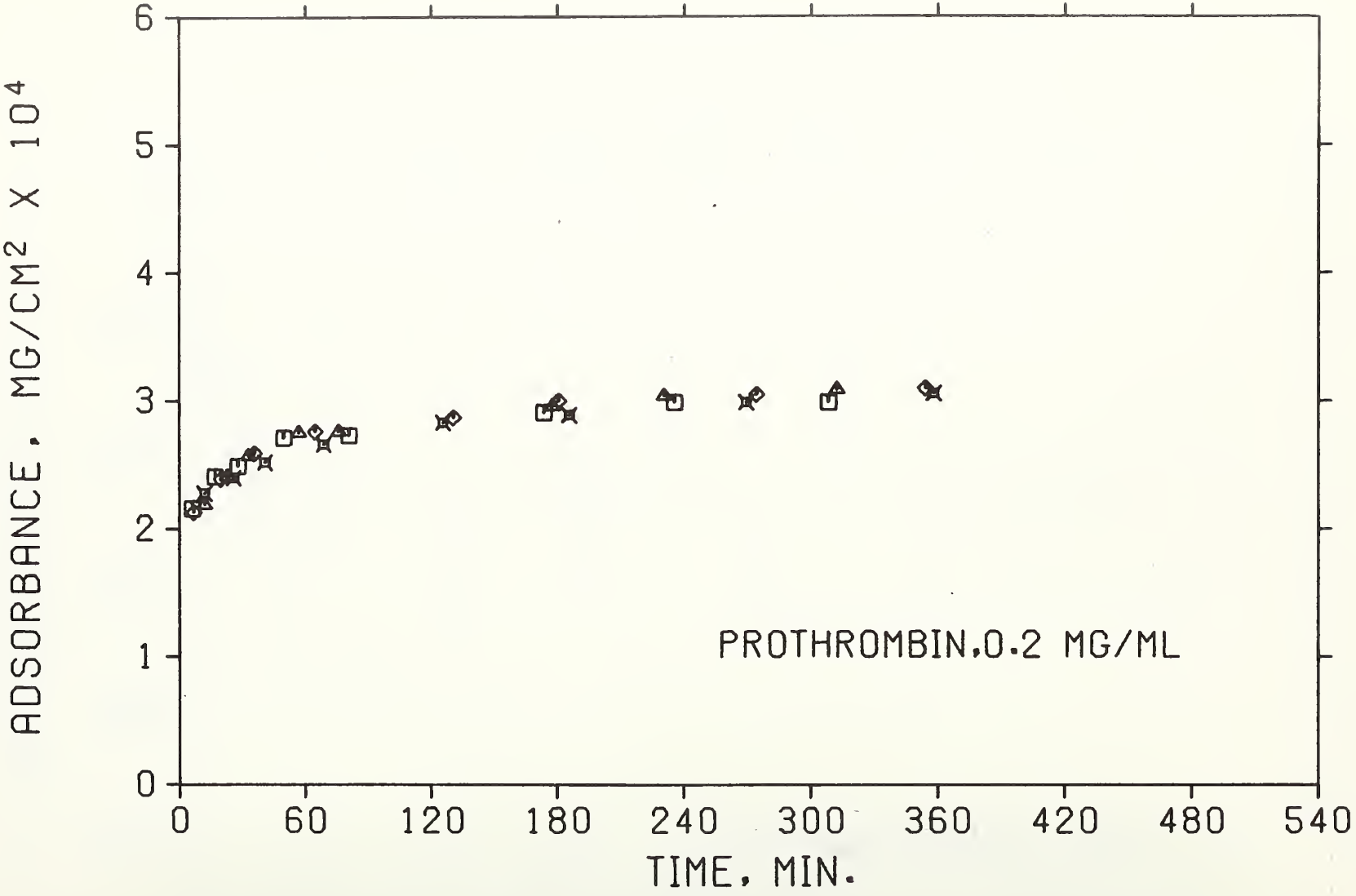

Figure 13: Adsorbance of prothrombin on chromium

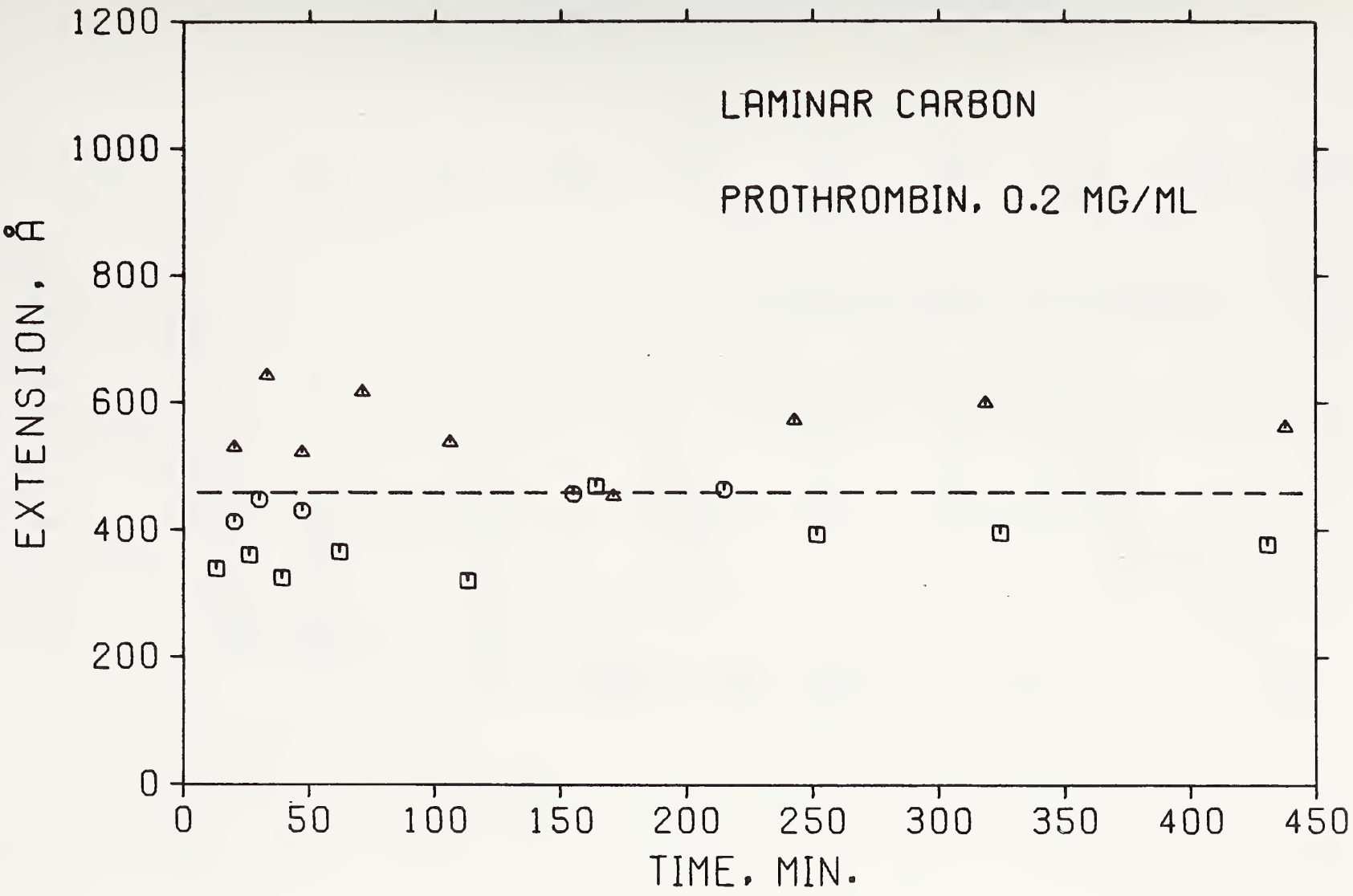

Figure 14: Extension of prothrombin on laminar carbon

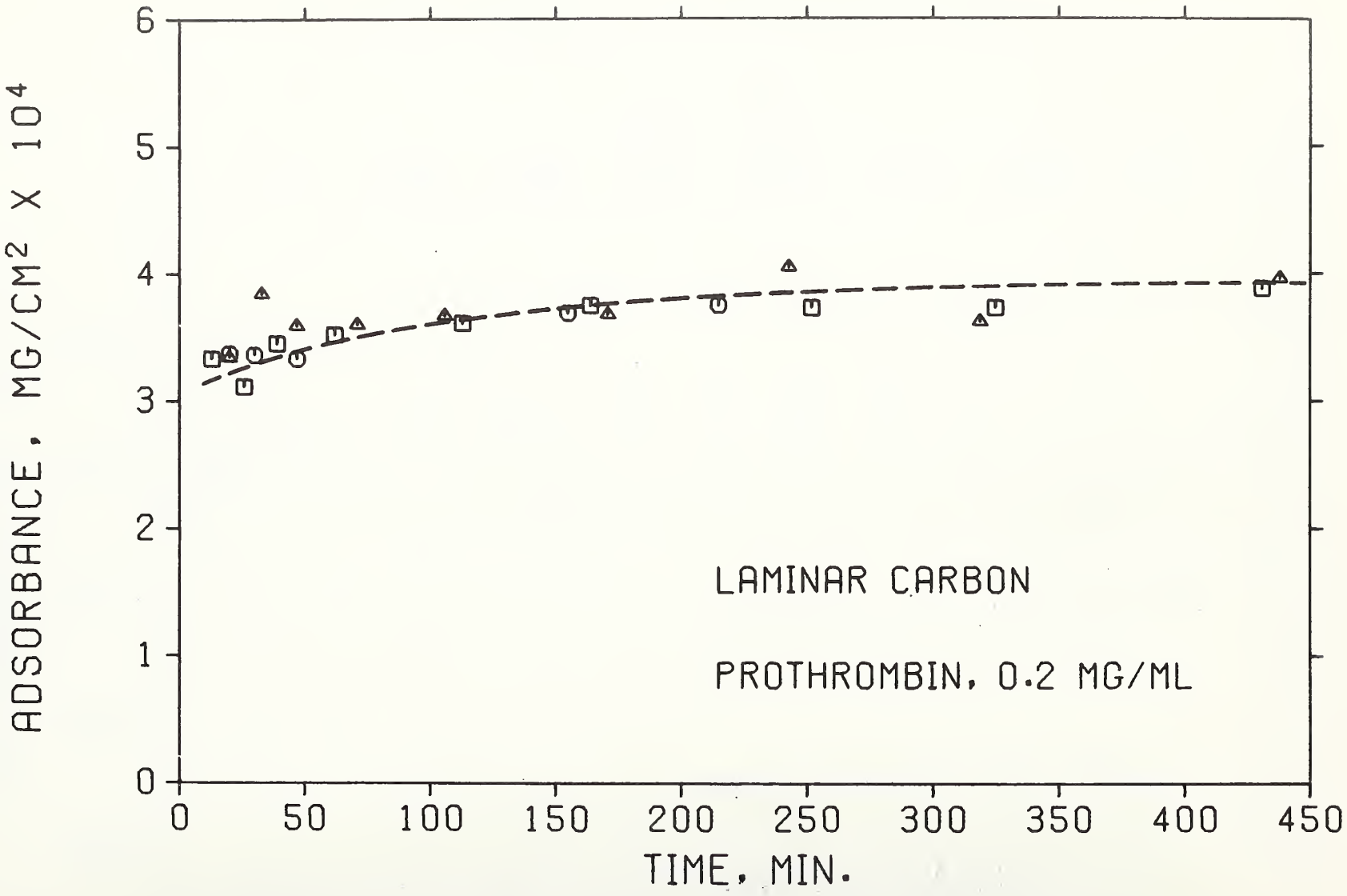

Figure 15: Adsorbance of prothrombin on laminar carbon

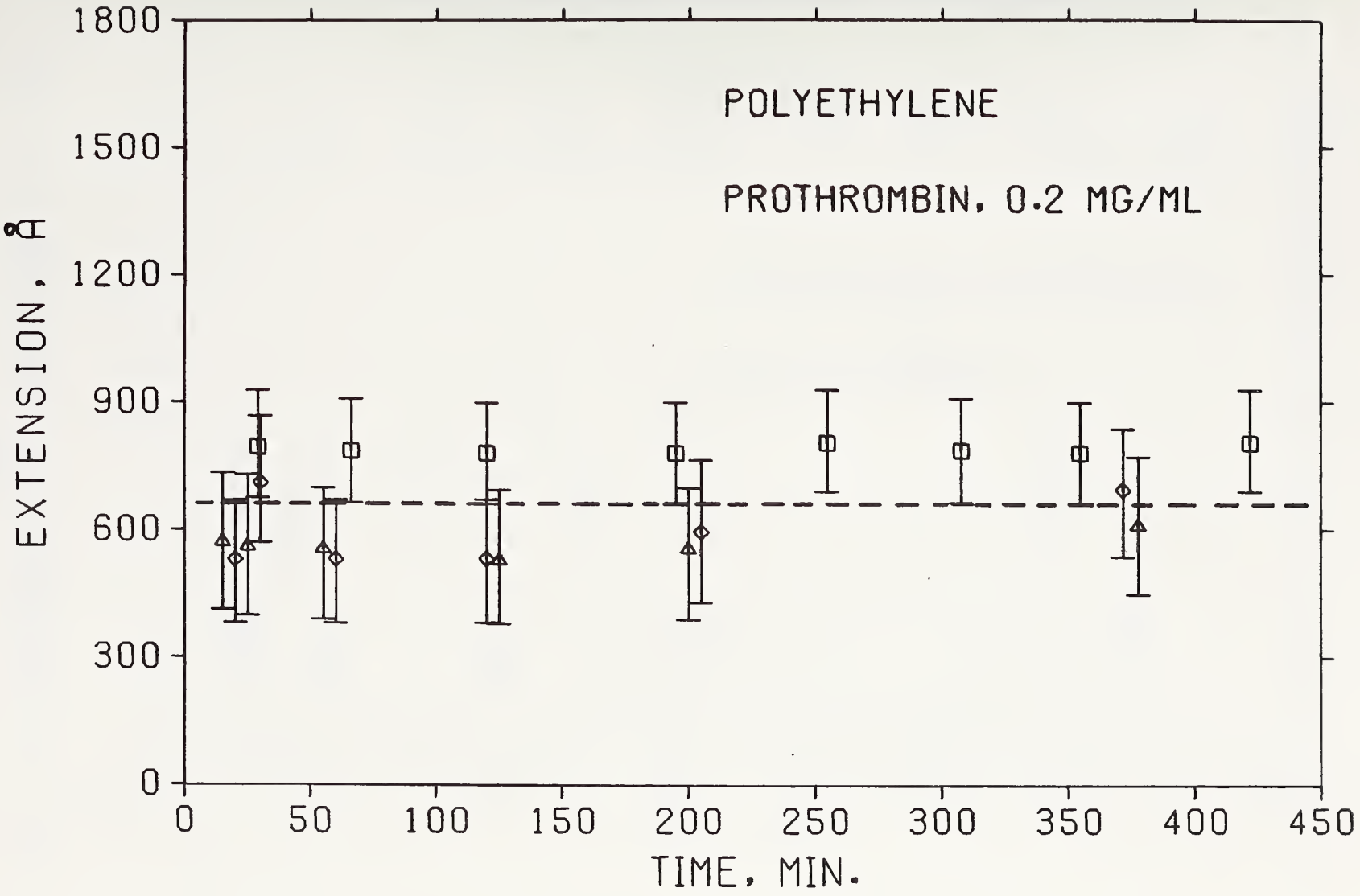

Figure 16: Extension of prothrombin on polyethylene

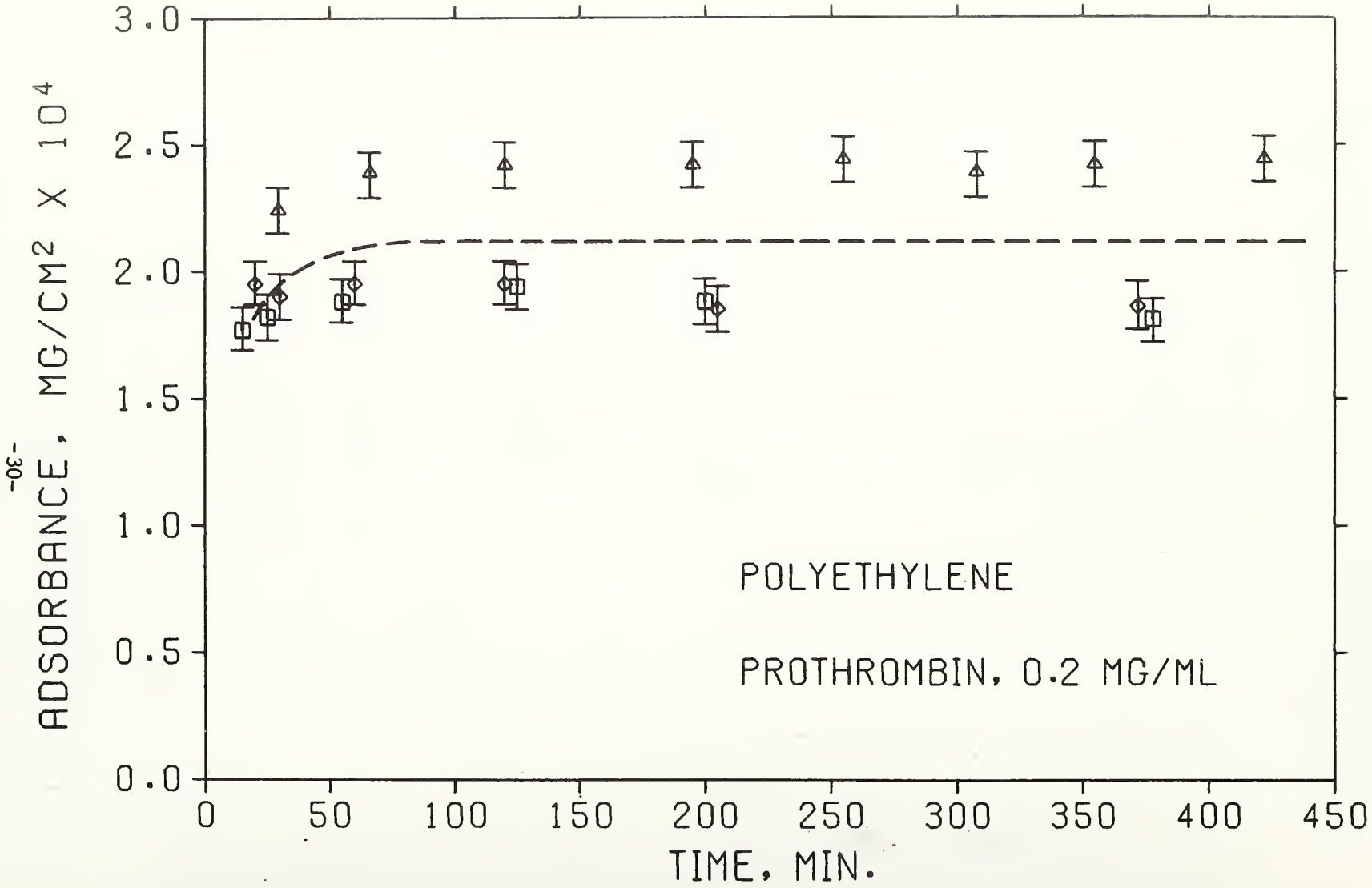

Figure 17: Adsorbance of prothrombin on polyethylene

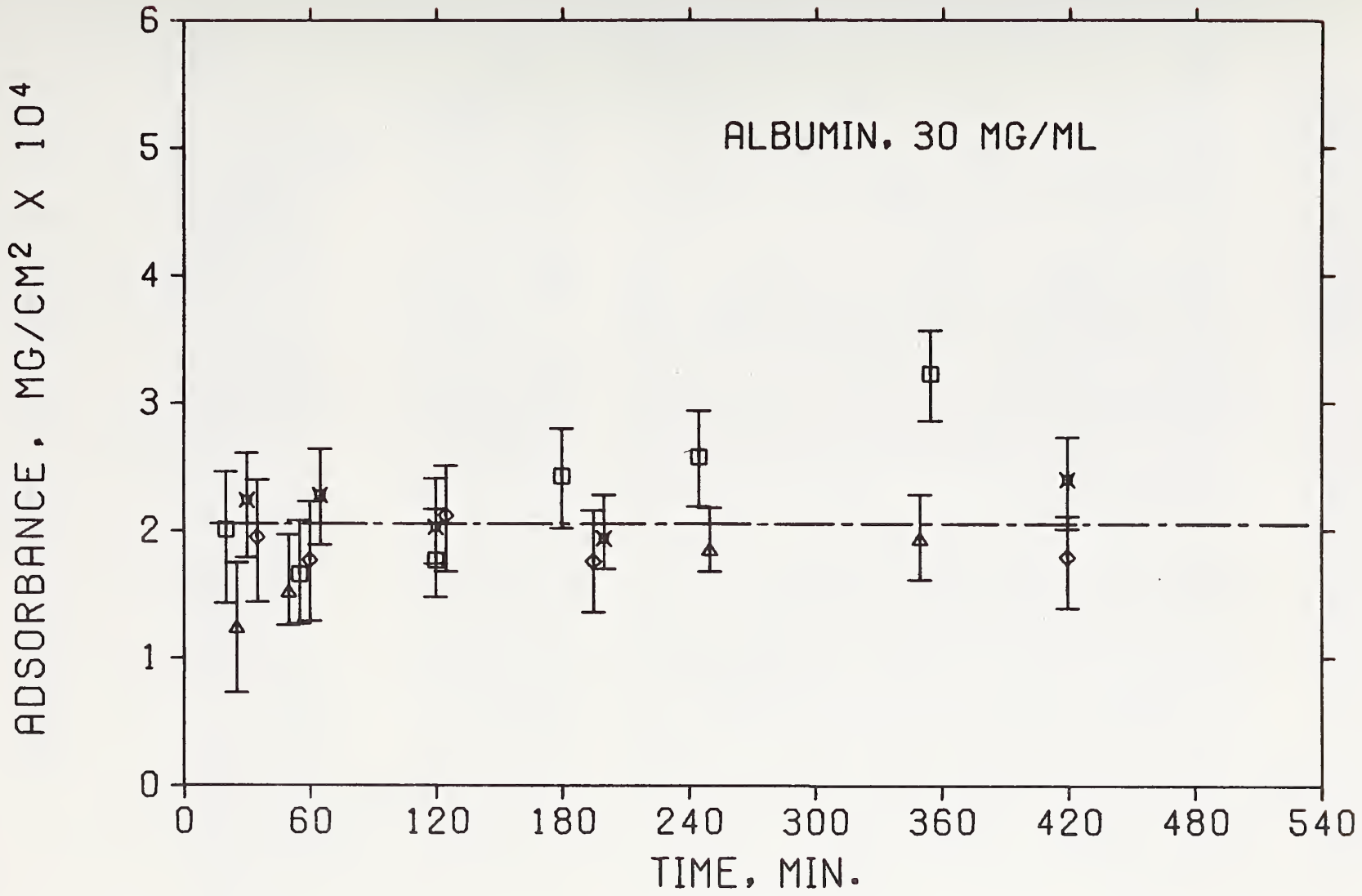

Figure 18: Adsorbance of albumin on chromium

fibrinogen although at long times extension and adsorbance values did not agree, within the error limits, for the two materials. On the lower energy carbon surface the adsorbance increased to a maximum and then decreased steadily as shown in Figures 19 and 20. An even more rapid decrease is seen on polyethylene (Figures 21 and 22) although readings after 400 minutes were imprecise because of the small amount of material left on the surface.

It can be speculated that this removal may be due to the action of plasmin, a fibrinolytic enzyme, which could come from the activation of plasminogen which is presumably a contaminant of Cohn Fraction I (47). Plasminogen contamination was surmised on the basis of short clot lysis times from the Fraction I fibrinogen. This explanation was tested in three ways. First, fibrinolysin, a factor elaborated by streptococcal bacteria which activates plasminogen, was added to the unpurified Fraction I and as shown in Figure 23, the rate of removal was increased. Secondly, ε-aminocaproic acid was added and was shown to retard the rate of removal (Figure 24). ε-Aminocaproic acid is an inhibitor of the plasminogen to plasmin interconversion and also a mild inhibitor of plasmin action (48). The Cohn Fraction I was purified by gel permeation chromatography under conditions reported to remove plasminogen (49) and the decrease was eliminated (open symbols in Figure 25). Fibrinogen purified by ammonium sulfate precipitation shows the same adsorbance behavior as the chromatographed fibrinogen but the clot lysis times are not as long. Finally, addition of fibrinolysin plus a small amount of unpurified fibrinogen to the purified fibrinogen restores the decrease (closed symbols in Figure 25). This evidence is not conclusive, but it is highly suggestive that the plasminogen-plasmin system is responsible for the removal of fibrinogen from the surface. It is interesting to note that the action of plasmin appears to be rather specific for the adsorbed fibrinogen as the supernatant solution remaining after almost all the fibrinogen has been removed from the surface still contains enough fibrinogen to be clotted by thrombin.

The reason for the lack of removal on the higher energy surfaces is not clear. It can be speculated that the plasmin is tightly adsorbed to the surface in these cases and therefore inactivated or that the fibrinogen is held in a conformation which hinders enzymatic attack. It may even be that proteolysis does indeed occur, but that the fragments are also held in these cases and no change in adsorbance is seen. We have no further evidence that bears directly on any of these contentions.

Equilibrium values

The equilibrium adsorbance and extension values for the proteins and surfaces studied are summarized in Table I. It should be emphasized that the different proteins were adsorbed at concentrations that approximate physiologic ones and are therefore quite different from one another. Albumin was adsorbed at 30 mg/ml, fibrinogen at 2.5 mg/ml and prothrombin at 0.2 mg/ml. The high albumin concentration gave a solution with a refractive index close

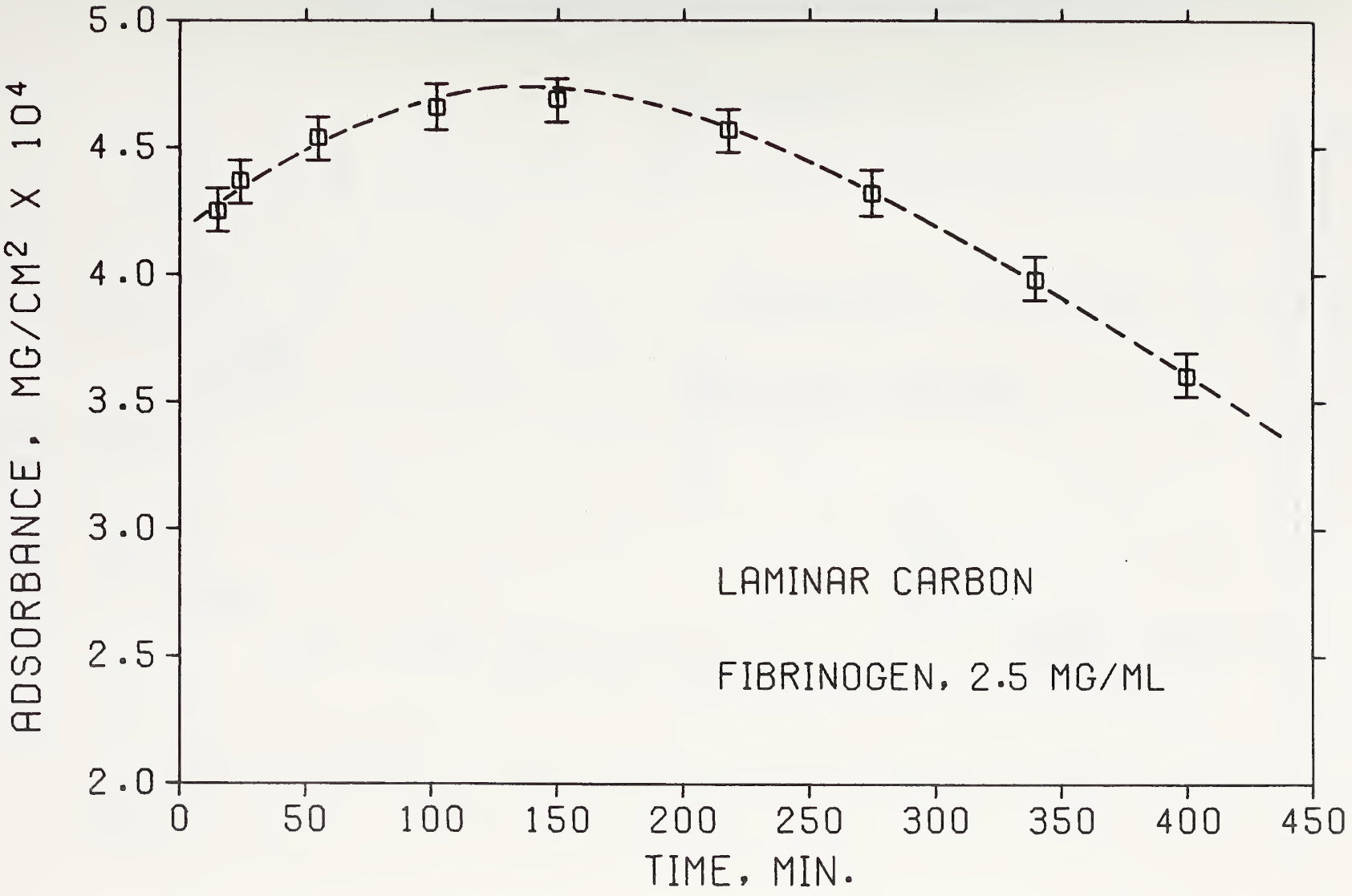

Figure 19: Adsorbance of Cohn Fraction I on laminar carbon

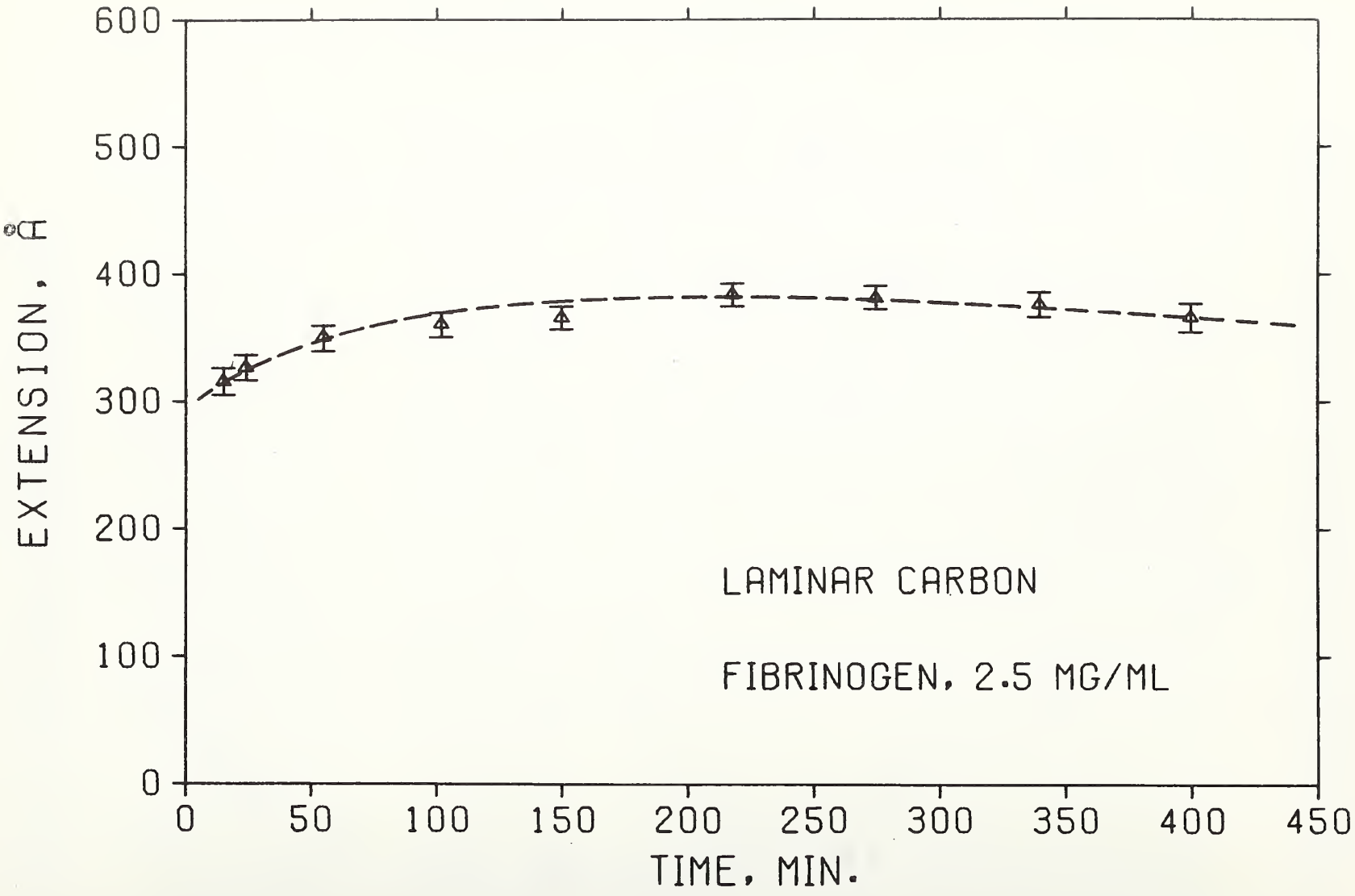

Figure 20: Extension of Cohn Fraction I on laminar carbon

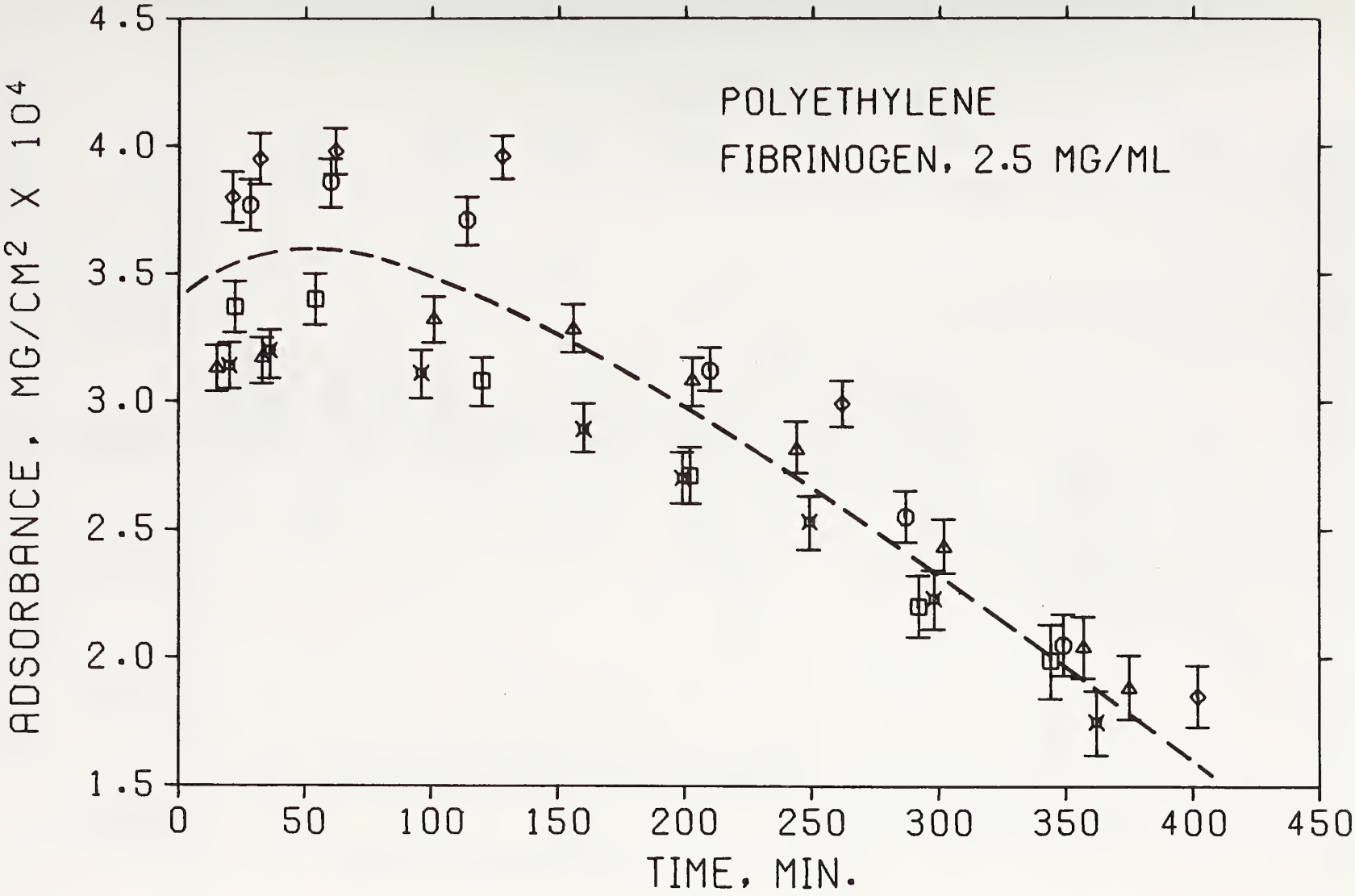

Figure 21: Adsorbance of Cohn Fraction I on polyethylene

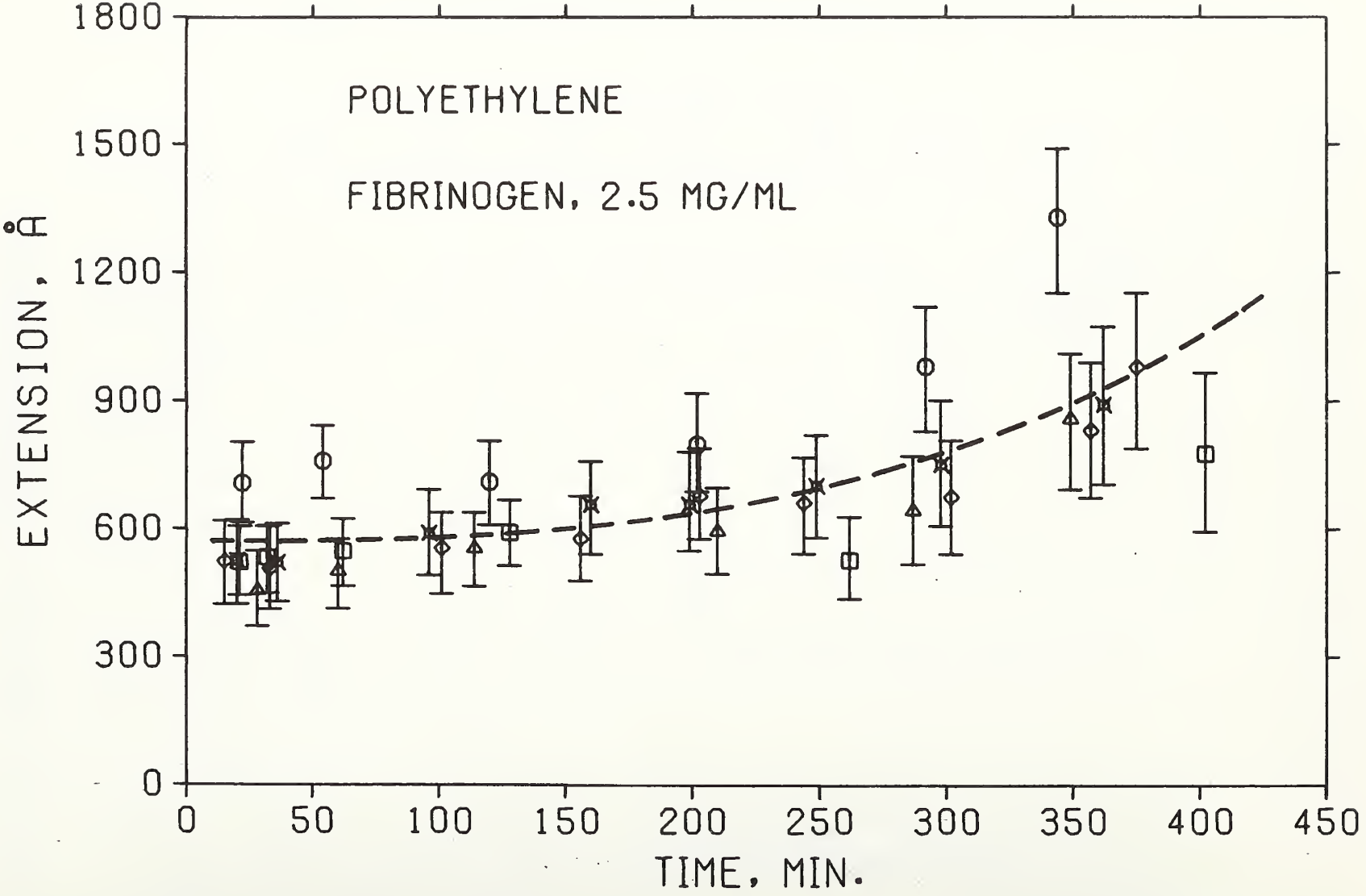

Figure 22: Extension of Cohn Fraction I on polyethylene

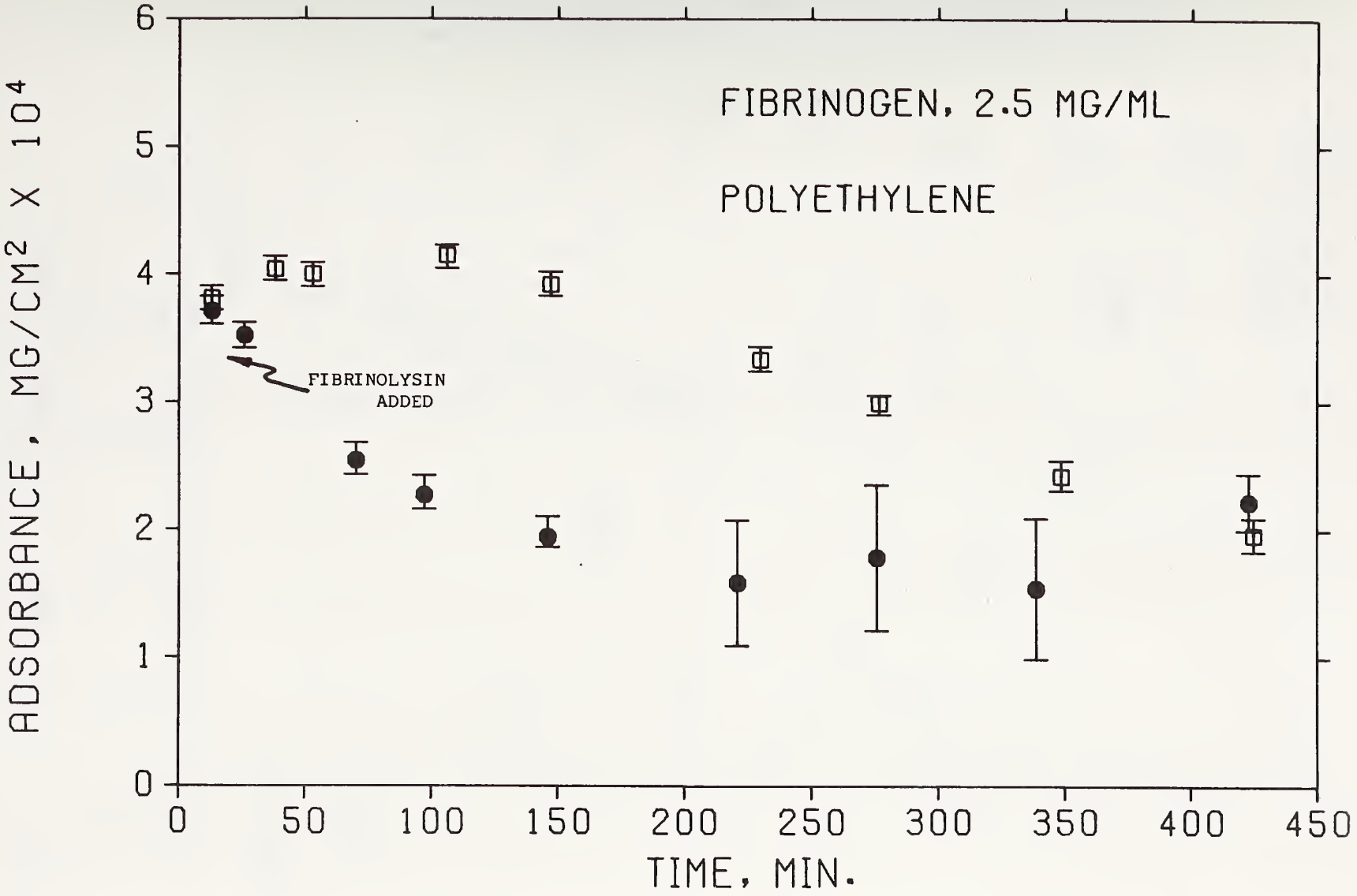

Figure 23: Adsorbance of Cohn Fraction I on polyethylene, open symbols; Cohn Fraction I with fibrinolysin added, closed symbols

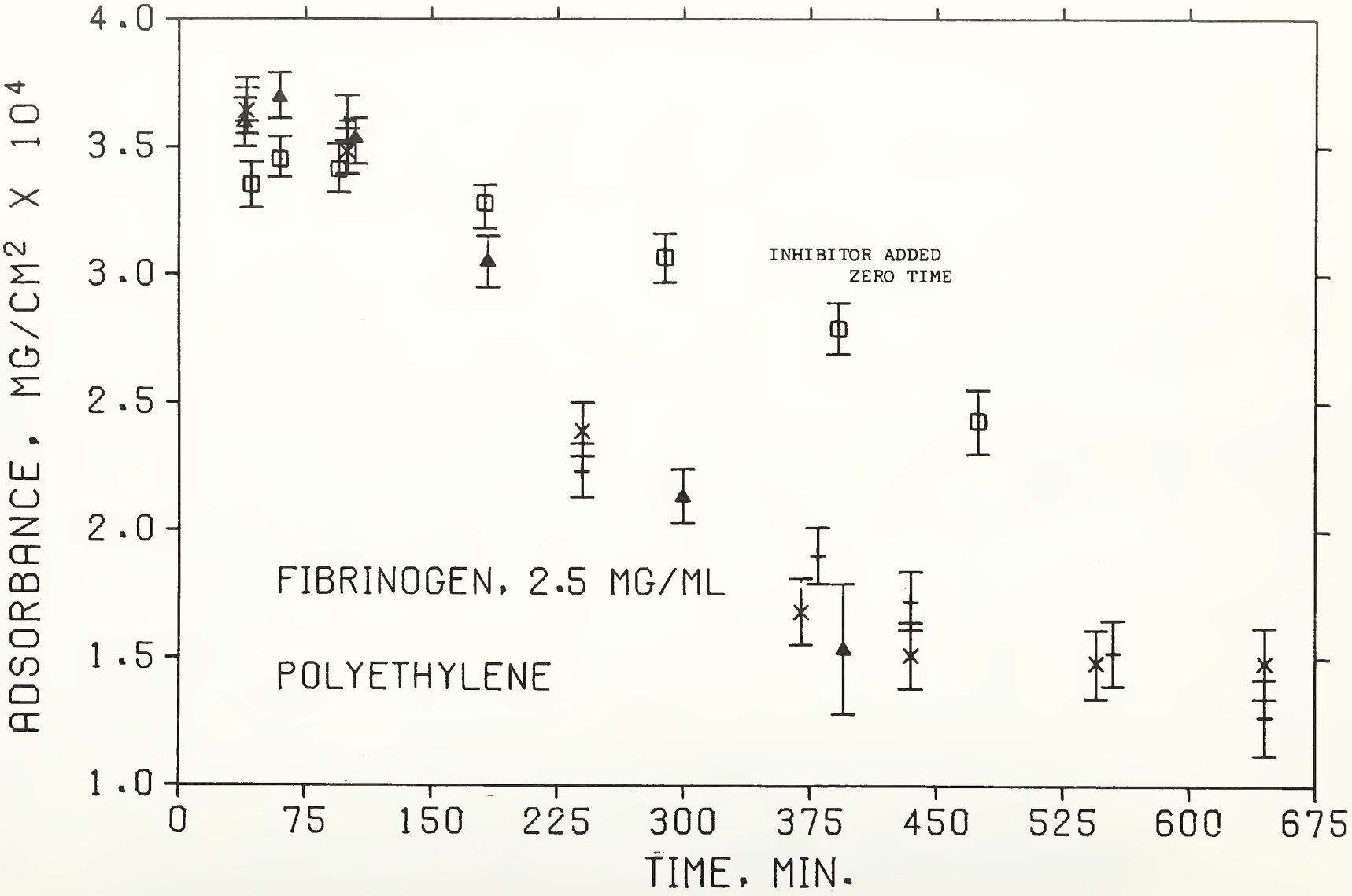

Figure 24: Adsorbance of Cohn Fraction I on polyethylene; Cohn Fraction I with ε-aminocaproic acid, open symbols

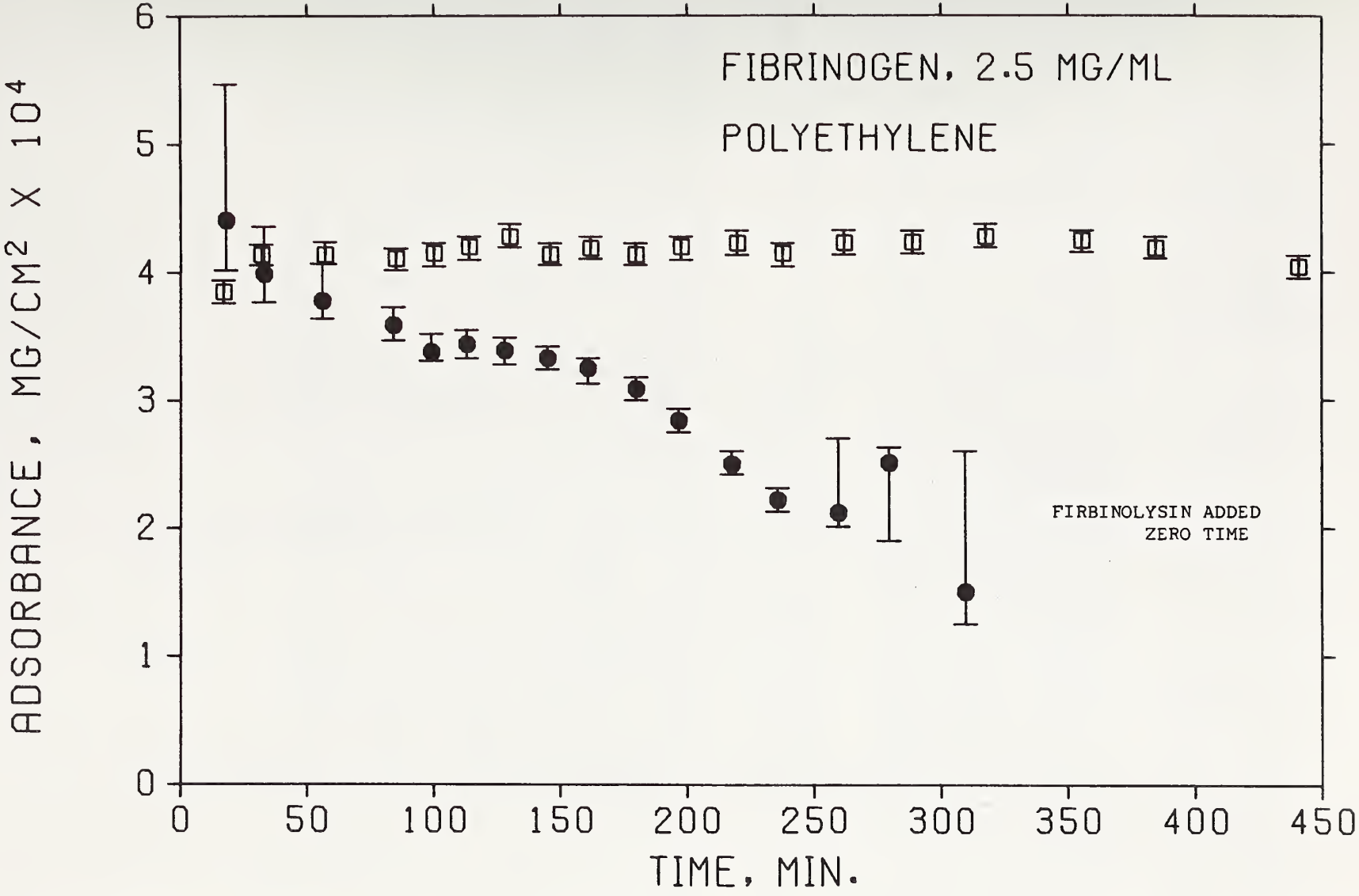

Figure 25: Adsorbance of purified fibrinogen on polyethylene, open symbols; purified fibrinogen with fibrinolysin and Cohn Fraction I added, closed symbols

to that of the adsorbed film. When this was coupled with a low substrate index, the resulting lack of sensitivity gave exceedingly wide error limits and meaningful measurements were not possible. This condition accounts for the blanks in Table I. Measurements can be made at much lower albumin concentrations, but they would then not be comparable to those on the high energy surfaces.

Two main features should be noted in the table. First, in spite of the large concentration differences in the proteins and the varied nature of the surfaces, the adsorbances cover only a rather small range. There is also no trend in adsorbance with surface energy. If surface energy is estimated by the critical surface tension for wetting, then the surface energies range from high to low in the series platinum, chrome, quartz, carbon, and polyethylene. There is, however, a correlation of surface energy with extension. Such an increase in extension with decreasing surface energy would be in accord with flat, tightly held conformations on the higher energy surfaces and looser, more extended conformations on the lower energy surfaces.

The adsorbances in Table I are all consistent with monolayers of protein of unperturbed molecular dimensions, but the concept of a monolayer may not be applicable to such large molecules.

Finally, the extensions given should not be construed as molecular dimensions of the adsorbed protein. The model assumed for the calculation of this extension is that of a film of homogeneous refractive index with discrete boundaries. Obviously, any large adsorbed molecule will show some refractive index inhomogeneities normal to the surface and this will affect the calculated extensions directly. This problem has been considered by McCrackin and Colson (27) who have shown that extensions calculated assuming a homogeneous film may be converted to root-mean-square segment to surface distances if the actual refractive index distribution is known. For example, the homogeneous film thicknesses have to be divided by factors of from 1.5 to 1.7 if the refractive index distributions are linear, gaussian, or exponential. As we have no estimate of the refractive index distribution of an adsorbed protein we have no way of making such a conversion for this data but the homogeneous extensions may perhaps be considered as upper limit approximations to molecular dimensions. The relative values of the extensions on the different surfaces may properly be compared assuming that the refractive index distribution does not change markedly from one surface to another.

Table I: Adsorbance, A, and molecular extension, d, of proteins on various surfaces as measured by ellipsometry.

	CHROME		PLATINUM		QUARTZ		CARBON		POLYETHYLENE	
	A, mg/m^2	d, Å	A, mg/m^2	d, Å	A, mg/m^2	d, Å	A, mg/m^2	d, Å	A, mg/m^2	d, Å
FIBRINOGEN	4.8	320	5.4	230	3.7	320	4.6	430	4.2	590
ALBUMIN	2.1	210	3.5	230	--	--	--	--	--	--
PROTHROMBIN	3.0	220	4.2	260	1.8	420	3.5	460	2.2	650

BOUND FRACTION

Equilibrium Conformation Studies

Effect of Surface Population - The isotherms of serum albumin, prothrombin, and fibrinogen are given in Figures 26-28. Figures 26 and 27 also include values of the bound fraction for serum albumin and prothrombin, respectively. Portions of the fibrinogen isotherm were inaccessible for bound fraction measurements using the technique described above because of the large adsorbance of this protein (initial concentrations were limited for fibrinogen because of the low solubility of fibrinogen in the D_2O - phosphate buffer).

For serum albumin and prothrombin, no systematic change in the bound fraction was apparent over the measured portion of each isotherm. The mean values with their computed standard deviations were 0.11 ± 0.02 and 0.11 ± 0.03 for serum albumin and prothrombin respectively. Results for fibrinogen showed an increase in the bound fraction with increasing amount adsorbed. In Figure 29 are shown the results of a set of four experiments carried out for the accessible portion of the isotherm. The dashed line represents a least-squares linear fit to the data. The unstable nature of the fibrinogen-silica suspension is at least partially responsible for the large scatter in the values of the bound fraction, p, and, in conjunction with the high adsorbance, prevented measurements of p at larger adsorbance values.

Using the known amino acid compositions of serum albumin, prothrombin, and fibrinogen, the bound fractions reported translate into a minimum of 77 contacts with the silica surface or serum albumin, 80 for prothrombin, and 176-703 for fibrinogen, as shown in Table II. If the adsorbed protein molecules were spread nearly flat on the surface, large values of the bound fraction would be expected. Adsorption of poly(ethylene o-phthalate) (31), poly(methyl methacrylate) (30), polystyrene (32), and poly(4-vinyl pyridine) (29) on Cab-O-Sil gave bound fractions of 0.37, 0.35, 0.24, and 0.35 respectively. In each of these cases, the synthetic molecules appear to lie close to the surface in a presumably spread condition. Since bound fractions of 0.11 were obtained for prothrombin and serum albumin, the adsorbed protein molecules are still relatively unchanged by the surface, despite the large number of attachments and great interaction.

The bound fraction data can yield information on changes in the conformation of the adsorbed protein molecule as a result of increasing competition for surface sites. A decrease in the bound fraction values with increasing population of protein atoms at the surface would be expected if changes in conformation were to occur. Such changes in conformation of synthetic random coil polymers have been observed (29,30,22). It has been suggested (50,51) that an adsorbed serum albumin molecule should initially unfold increasing the value of p, which would presumably then decrease with increasing surface population. Our results for serum albumin and prothrombin, Figures 26 and 27, show that the bound fraction remains constant, indicating that the conformation does not change as the surface population increases. The relatively low value of p for these proteins as compared to synthetic random coil polymers, and the constant value of p over the isotherms, strongly suggests that the internal bonding and disulfide cross-links of the globular proteins are sufficient to preserve the basic tertiary structure of the molecules.

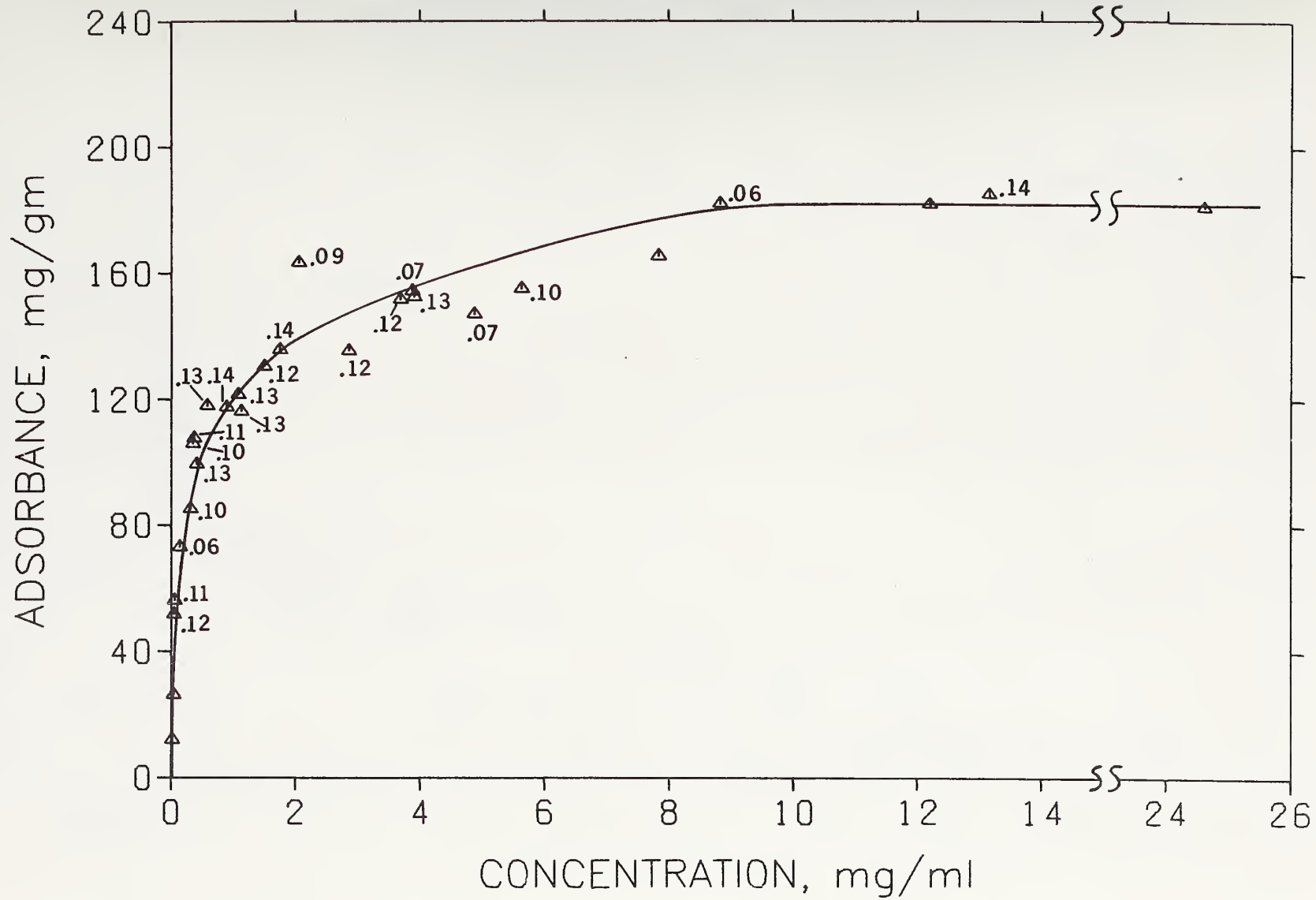

Figure 26: Adsorption isotherms for bovine serum albumin at pD 7.4 on silica. Values of bound fraction given for isotherm points.

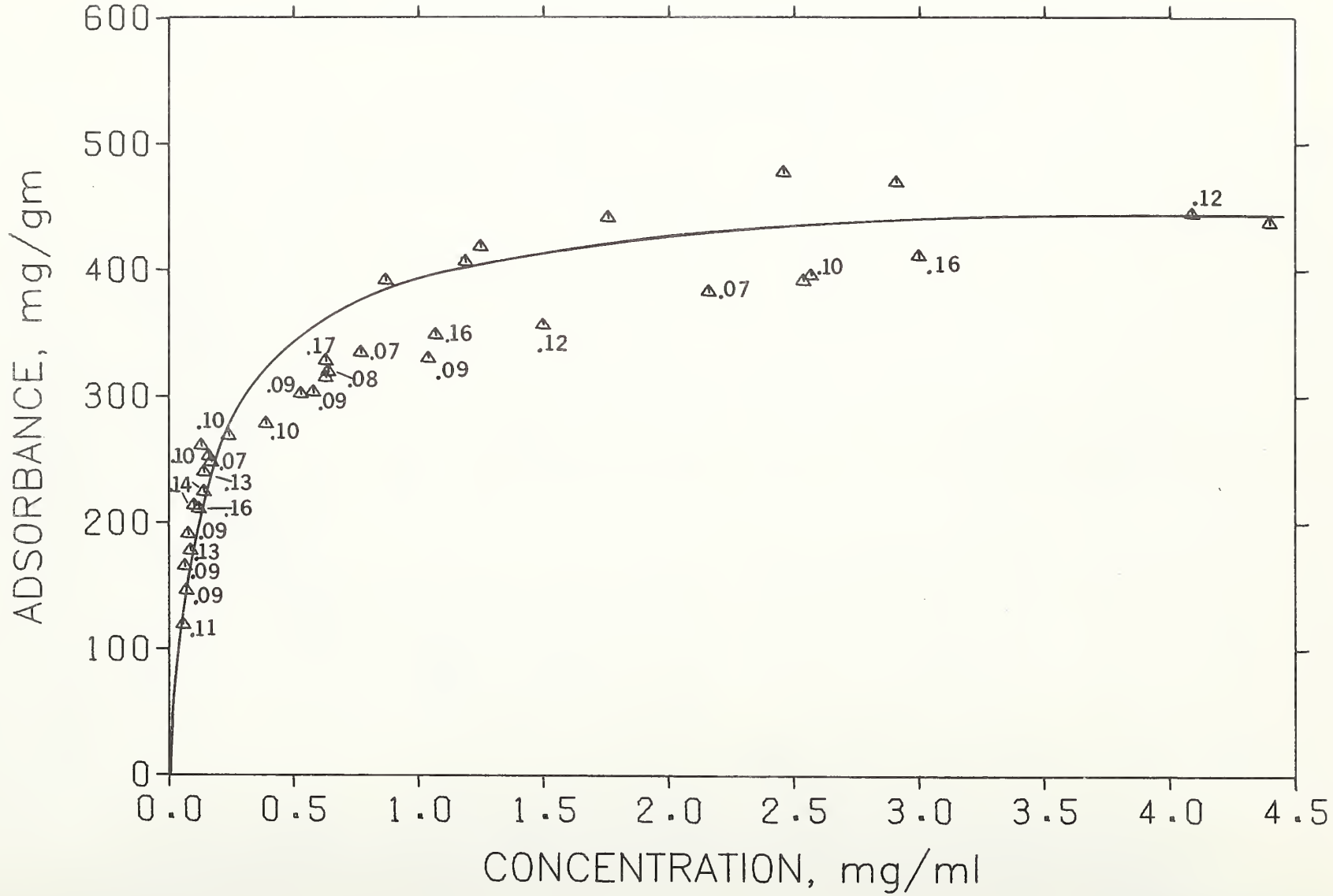

Figure 27: Adsorption isotherm for bovine prothrombin at pD 7.4 on silica. Values of bound fraction given for isotherm points.

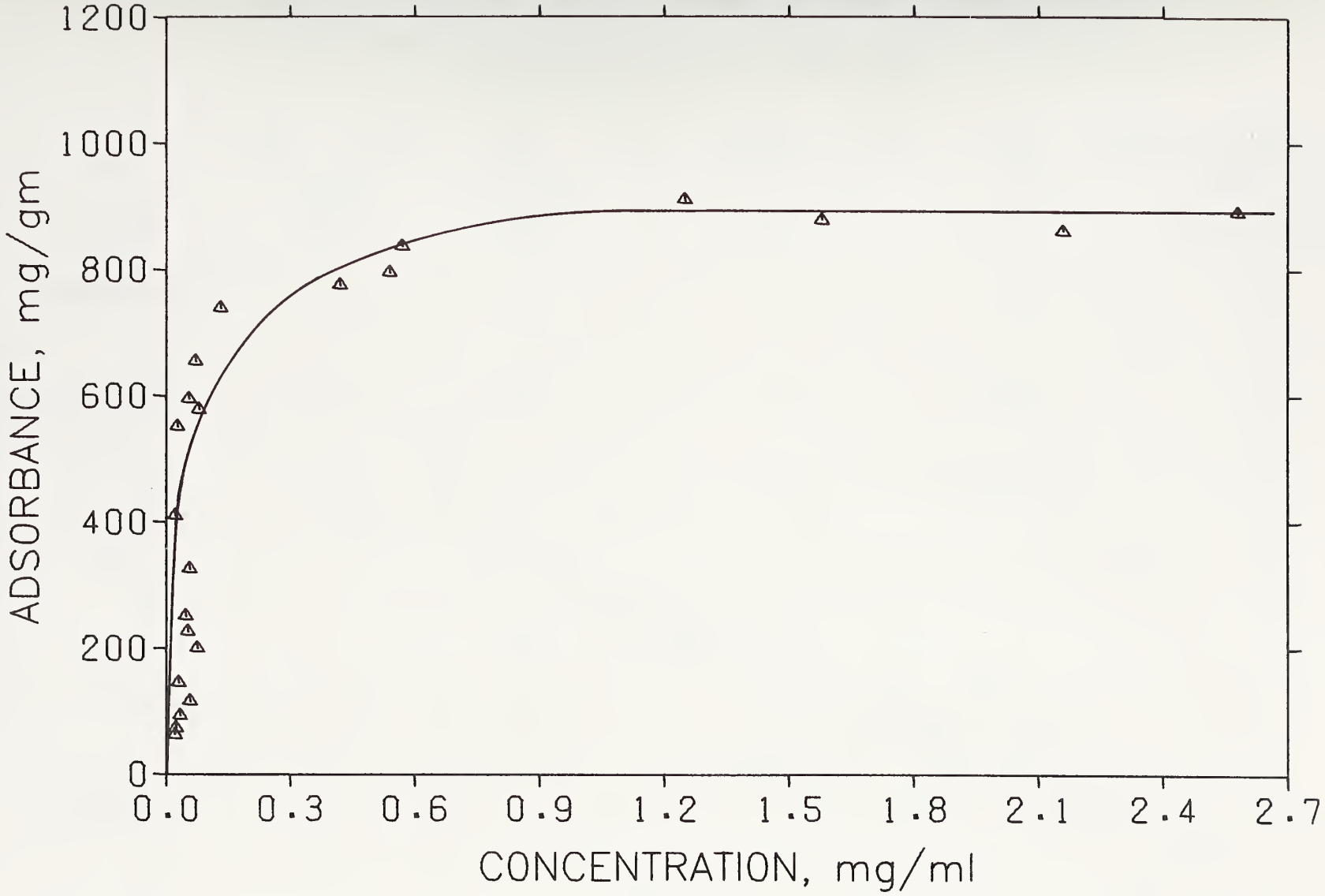

Figure 28: Adsorption isotherm for bovine fibrinogen at pD 7.4 on silica.

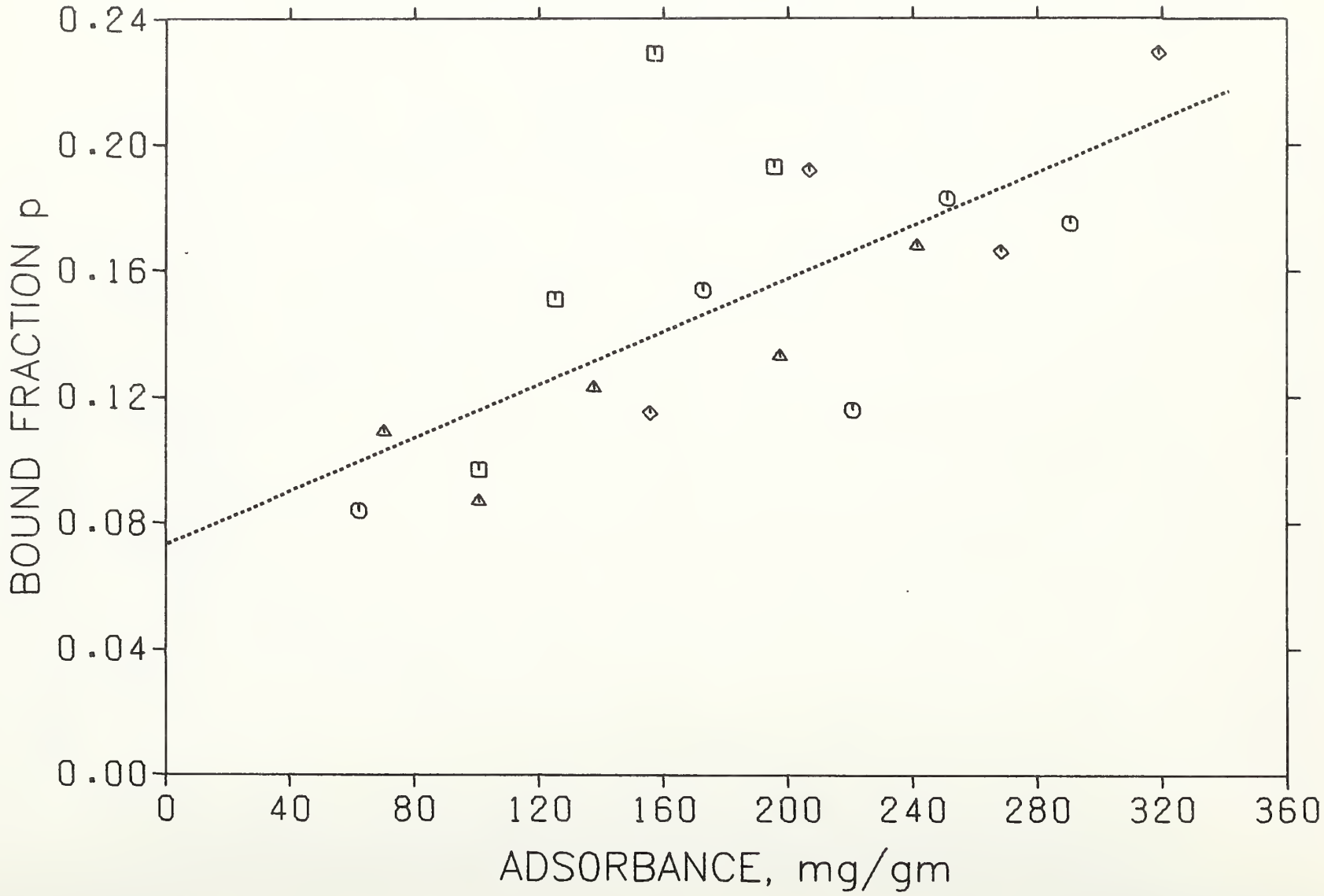

Figure 29: Bound fraction vs. adsorbance of bovine fibrinogen for four different experiments. Each symbol represents a separate run. Adsorption at pD 7.4 on silica.

Table II: Summary of Adsorption and Bound Fraction Data for Proteins

Protein	Maximum Adsorbance mg/gm	Maximum Adsorbance mg/m^2	Equil. Conc. for Maximum Adsorbance mg/ml	Bound Fraction	No. Carbonyl Contacts per Molecule with Surface
Serum Albumin	180	0.88	11	0.11	77
Prothrombin	440	2.16	4.0	0.11	80
Fibrinogen	890	4.36	1.0	0.05-0.20	176-703

The adsorption behavior of fibrinogen (Fig. 29), which shows a direct dependence of bound fraction on adsorbance, does not necessarily represent a conformational change. As noted above, previous experience with synthetic polymers has shown that conformation changes resulting from an increase in surface population are accompanied by decreases in the bound fraction. The bovine fibrinogen molecule is rodlike with a length of between 400-600 A (52) and can easily interact with more than one silica particle. It has been postulated (53) that the solubility of fibrinogen is due to electrostatic repulsion between regions of high negative charge density localized in fibrinopeptides A and B. If adsorption and interaction with the hydrophilic surface were to effectively neutralize this repulsion, fibrinogen molecules adsorbed to different silica particles could interact like fibrin, thereby increasing the surface attachments of each fibrinogen molecule. Such an interfacial aggregation (51) could result in an increase in bound fraction values with increasing adsorbance, without significantly hindering further adsorption to the remaining available surface.

Our data for maximum adsorbance can be converted to amounts/unit area using the measured surface area. (Surface area determinations of Cab-O-Sil using stearic acid adsorption (54) agree with the BET-N_2 analysis). These results are presented in Table II and agree quite favorably with those found for adsorption of human blood proteins on quartz using ellipsometric techniques as shown in Table I. Results obtained by MacRitchie (51) for adsorption of bovine serum albumin on a silica surface (also 200 m^2/gm) at pH 7.5 were only one third as great as that found in the present study. The reason for this difference is not obvious.

Variation of pD and Ionic Strength - Experiments were conducted to determine the effect of pD and ionic strength on the adsorbance and bound fraction of serum albumin. The results obtained for an initial concentration of 4.3 mg/ml serum albumin at ionic strengths of 0.1, 0.2, and 0.5 in D_2O-phosphate buffer are presented in Figure 30. The curve drawn represents the effect of pD on adsorbance for ionic strength 0.1. The figures given along the curve are the values of the bound fraction at that pD. Since no effect of ionic strength was observed on the bound fraction within experimental error, the values of p are the average for the three ionic strengths. Both the adsorbance and bound fraction reach a broad maximum at approximately pD 5.2 for ionic strengths of 0.1 and 0.2. For these two values of ionic strength, there is no effect on the adsorbance at or near the maximum, while strong effects are noted at the extremes of pD. The pD profile of adsorbance for I = 0.5 shows no maximum, although there appears to be an asymptote in the acid region. A maximum in the bound value also occurs near pD 5.2, although the profile is different from that of the adsorbance vs pD curve. While the adsorbance values at pD 7.4 are near the plateau values, those at pD 5.0 are only on the knee of the isotherm (plateau adsorbance at pD 5: A = 308 mg/gm at C_{equil} = 3.0 mg/ml).

The adsorbance-pD profiles of serum albumin on silica are similar to those observed by Bull (55) on glass. Silica exhibits a decreasing negative charge over the pH region of 8.0 to 2.0 as determined from electrophoretic mobility (56,57) and sedimentation (58) methods. If electrostatic attractions between the adsorbing protein and silica were the factor governing adsorption, the adsorbance should increase as the pD decreases, since the isoelectric point of serum albumin occurs at pD 5.0. The conformation of bovine serum albumin in solution undergoes interesting changes with pH (59). At values acid to the isoelectric point, the molecule reversibly unfolds and becomes flexible, until

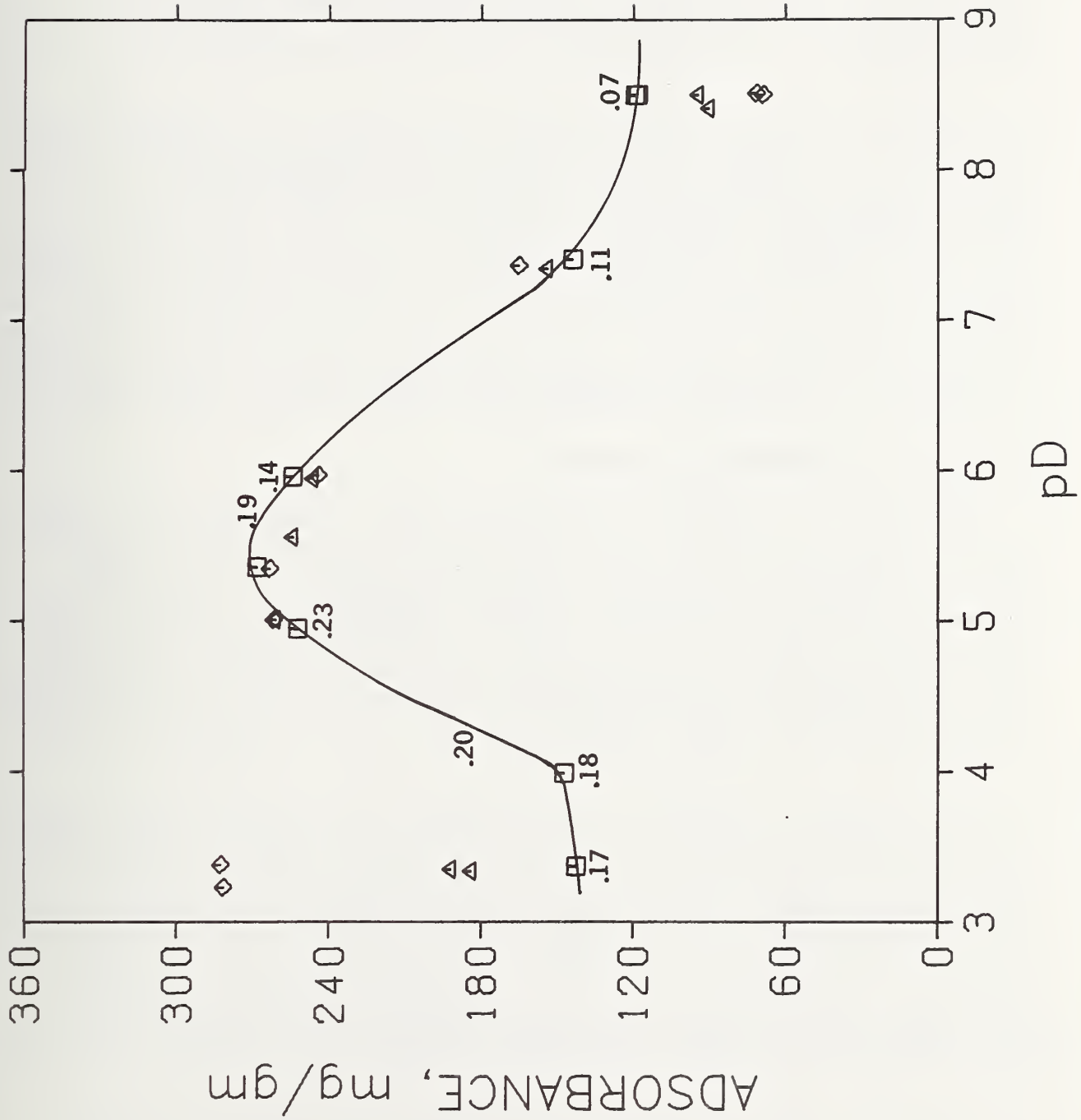

Figure 30: Adsorbance vs. pD for bovine serum albumin at 0.1 (⊡), 0.2 (▲), and 0.5 (◇) ionic strength on silica. The curve represents ionic strength 0.1. The values given on the curve represent the bound fraction at a given pD. These values were the same for all three ionic strengths.

finally at pH 2.5 - 4.0 with I > 0.1, an aggregation occurs (60). The experimentally determined bound fraction (Fig. 30) as a function of pD is, therefore, the result of a number of contributing effects which cannot now be assessed. The coincidence of the maxima near pD 5 of the adsorbance and bound fraction profiles strongly suggests, however, that interactions between the adsorbed protein molecules are important in determining both the adsorbance and conformation. This is consistent with the results obtained in general for adsorbing polyelectrolytes (61).

Relating the plateau values of protein isotherms for a solid-liquid system with a close-packed monolayer can be very misleading. Using accepted figures for the molecular geometry of serum albumin in solution (62), one would calculate an adsorbance for our system of 520-1460 mg/gm depending on whether the molecule is adsorbed side-on or end-on. We do not approach these values under any conditions. At pD 3.35, the plateau adsorbances for ionic strengths of 0.1, 0.2, and 0.5 are 142, 193, and ~365 mg/gm, respectively. The bound fraction (conformation) is, however, not a function of the ionic strength (Fig. 30). One would, therefore, predict identical plateau adsorbance values for each ionic strength. It is clear that caution must be exercised in interpreting the available surface area per adsorbed molecule as an indication of changes in conformation upon adsorption.

Non-Equilibrium Conformation Studies

Dependence of Conformation on Adsorption Time - The possibility that the conformation of the adsorbed protein might vary as a function of the length of time for adsorption was tested by determining the bound fraction for each protein periodically following the formation of a suspension. Figure 31 illustrates some typical results obtained for serum albumin, prothrombin, and fibrinogen where the bound fraction values are listed over each curve at the time of measurement. Although the rates of adsorption cannot be compared since the initial conditions are different for each experiment, it is clear that there is no variation in the bound fraction for any of the proteins over the time period investigated at these conditions.

Our studies of the effect of length of time for adsorption on the bound fraction indicate that molecular rearrangements do not occur following adsorption at the concentrations studied. Although the adsorption rates for prothrombin and fibrinogen were extremely high as a result of the conditions required for bound fraction measurements, the relatively slow adsorption of serum albumin allows measurements of p as a function of time which shows that the conformation is not dependent upon the surface population, as was found in the equilibrium adsorption studies (Figs. 26 and 27). The slight decrease in adsorbance of prothrombin with time shown in Fig. 30 is possibly caused by the dissociation of an aggregate at the low final solution concentration. Prothrombin has been reported (63) to associate reversibly at low ionic strengths.

Comparative Rates of Adsorption - To compare the rates of adsorption of serum albumin, prothrombin, and fibrinogen, each was adsorbed at an initial concentration of 1.75×10^{-5} M with 0.004 gm silica per ml protein solution. The rates of adsorption are quite different, as shown in Figure 32. These

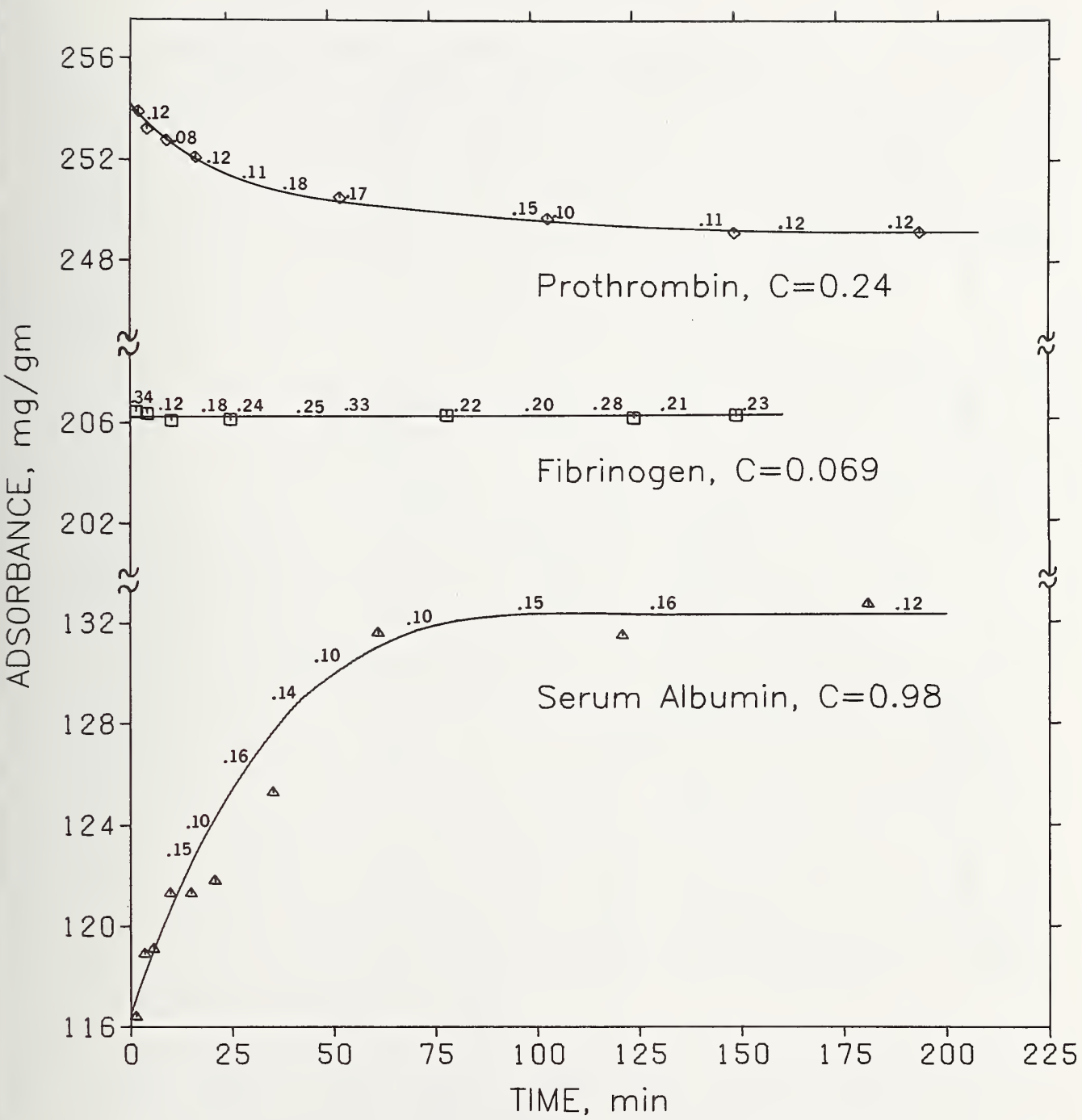

Figure 31: Adsorbance vs. time for bovine serum albumin, prothrombin, and fibrinogen. Values of bound fraction are given over each curve at the time measured. Adsorption on silica at pD 7.4.

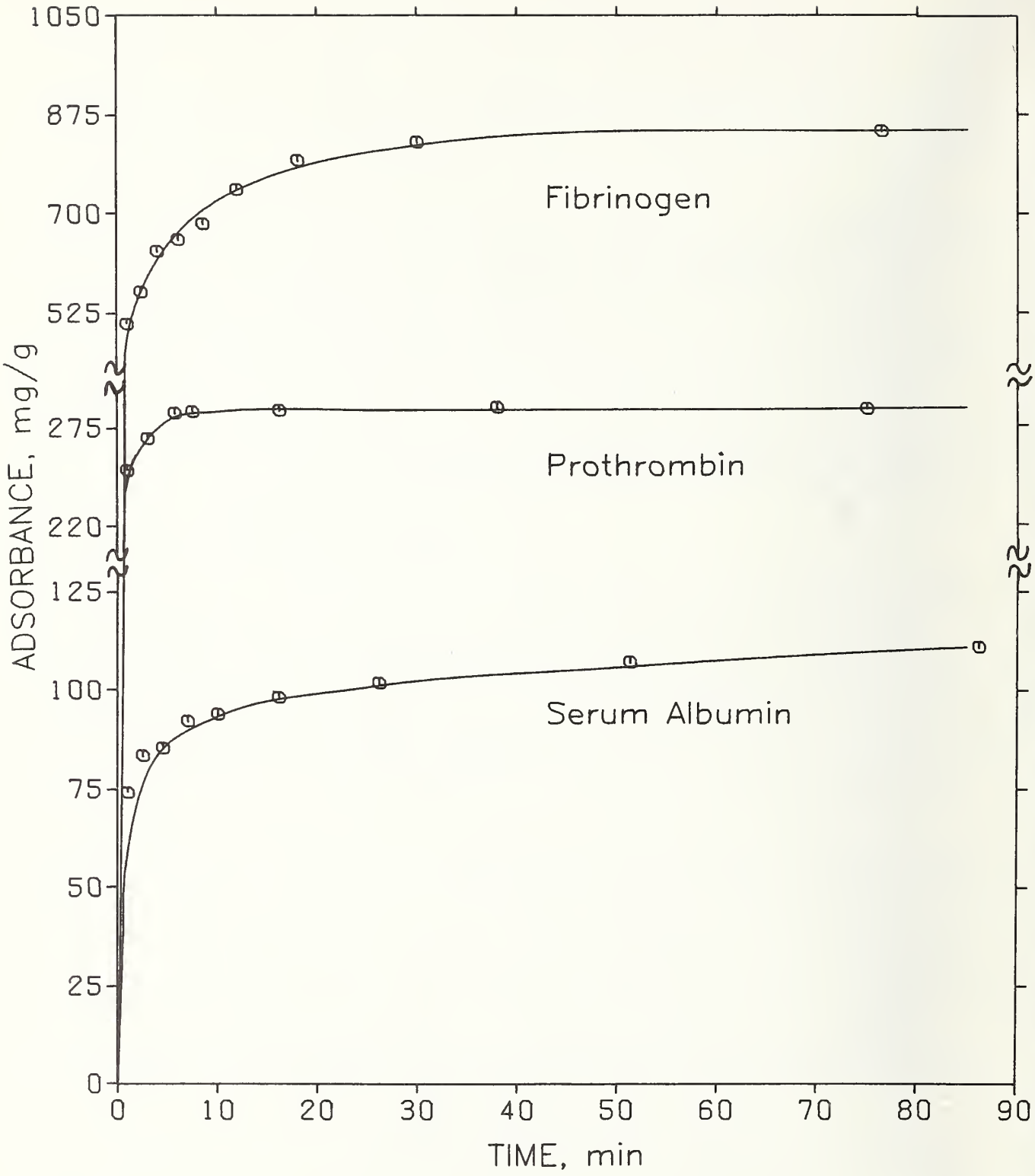

Figure 32: Adsorbance vs. time for 1.75×10^{-5} M bovine serum albumin, prothrombin, and fibrinogen. Adsorption on silica (0.004 gm/ml solution) at pD 7.4.

differences are more apparent when the data is presented as the ratio of the adsorbance at a given time to the value at the plateau, as is shown in Table III. Clearly prothrombin is more rapidly adsorbed for all time intervals. Serum albumin and fibrinogen, while very similar in their rates at early times, differ markedly in their approach to equilibrium.

The findings of the comparative rate study for the three proteins on silica are very similar to the rates observed by Lyman (64) for adsorption of the same proteins on flat polymeric sheets. To determine if the adsorption process is diffusion controlled, the results we obtained for fibrinogen were subjected to an analysis described by Crank (65) for adsorption on spheres from a solution of finite volume and known initial concentration. Using the nominal diameter of 120 A of Cab-O-Sil as the particle size and a diffusion coefficient of 2.02×10^{-7} cm^2/sec for fibrinogen, one calculates that adsorption should be complete in 5×10^{-7} sec. It is apparent that factors other than diffusion must govern the adsorption of the proteins studied on a finely divided silica.

Changes in Conformation of Serum Albumin Upon Adsorption

The conformational changes of blood proteins upon adsorption are extremely important to any understanding of surface induced coagulation. A priori, one does not know how much denaturation, if any, occurs upon adsorption of native proteins. The effects of any such denaturation are necessarily included in our values of the bound fraction. The presence of an infrared shift in the difference spectrum for the bound carbonyl group does not necessarily mean that protein denaturation has occurred upon adsorption. The shift may occur for adsorbed protein, even if the tertiary structure has not changed. It is possible, however, with the aid of certain assumptions to gain some insight regarding the amount of distortion of the molecule that occurs upon adsorption.

The observed bound fraction for serum albumin can be considered to be the sum of two contributions: the first due to contacts permitted by the native tertiary structure with the surface and the second resulting from surface induced rearrangements that occur as a consequence of adsorption. These contributions can be separated by determining the bound fraction of a highly cross-linked, and therefore rigid, material. Cross-linking of serum albumin with diethyl malonimidate dihydrochloride (43) produces a protein which is resistant to denaturation, has the same electrophoretic mobility, and most important, retains the same antigenic activity as native serum albumin. We prepared such a cross-linked material which had 45% of its free amino groups blocked (66). The average result of six experiments show in Table IV gave a bound fraction which was experimentally indistinguishable from that of the native protein. We conclude that surface induced rearrangements, if present in native serum albumin, are small.

As a means of assessing the extent of denaturation of native material upon adsorption, we have denatured serum albumin with known, mild techniques and determined the bound fraction of the resulting material. Table IV lists the results for heat denaturation at 66°C and thioglycollic acid reduction. It is clear that the bound fraction for a given protein is strongly dependent

Table III: Fraction of Protein Adsorbed

Protein	1 min	5 min	Time to Plateau, min
Prothrombin	0.88	0.94	8
Fibrinogen	0.60	0.76	75
Serum Albumin	0.64	0.76	170

Table IV: Bound Fraction of

Modified Serum Albumin

Sample	Bound Fraction
Native (Control)	0.11
Cross-linked	0.10
Heated at 66°C (2-60 min)	0.04
TGA Reduced	0.19

on its tertiary structure. Heat, which is known to cause the irreversible aggregation of serum albumin (67), leads to a bound fraction one third that of native material. Mild reduction, where one disulfide bond was broken as determined by a p-chloromercuribenzoate titration (41), leads to a bound fraction of about 1-1/2 times that for native protein.

These studies also shed light on the conformation of native adsorbed serum albumin. If the native protein were unfolded by an amount equivalent to the breaking of one disulfide bond, an increase of 55 contacts (Tables IV and II) would result. On the other hand, pretreatment of the serum albumin in a manner which has been reported (67) to result in aggregates resulted in a decrease of 50 surface contacts, implying that no aggregation occurs upon adsorption of the native serum albumin. These results for denatured serum albumin show that bound fraction is sensitive to what in our view are small conformational changes.

Conformation Studies of Model Compounds

Experimental and Computer Simulation of Lysozyme Adsorption - In order to understand better the extent of the changes in protein structure which may occur upon adsorption, computer simulation and experimental data were compared for a protein of known structure, lysozyme. Accordingly, bound fraction measurements were made at two different initial concentrations of lysozyme where the ratio of lysozyme molecules to silica particles (assuming a 120 Å diameter) was 5.5 and 14.2. The experimental value of p = 0.12 thus determined is equivalent to 19 carbonyl groups using the known amino acid composition of lysozyme (68). The algorithm described in the technique section was then applied to the known coordinates, and the minimum distance for 19 carbonyl oxygen atoms from the adsorbing interface determined by inspection of the output for all possible orientations. With respect to the coordinate system established, this distance was 3.00 Å and occurred at angles of rotation of 124° and 0° about the X and Y-axes respectively. Figure 33 gives the distribution of carbonyl oxygen atoms near the surface for this particular adsorbed orientation. While the minimum distance for 19 carbonyl oxygen atoms is approximately 3 Å from the interface, the mean distance assuming random orientation is 7.62 Å.

These results can be used as a model to gain some insight into conformational changes accompanying adsorption. For the interaction distance of 1.88 Å [the N-H-N hydrogen bond distance for nucleic acids minus the N-H bond length (69)], only 13 carbonyl oxygen atoms compared with the 19 determined experimentally are found to be interacting with the surface (Fig. 33), indicating a small (with respect to changes in the radius of gyration) rearrangement upon adsorption. One could also attempt to use a two state model and treat the observed bound fraction as a linear combination of some fraction of totally native and denatured material. (Bound fraction parameters describing the native and denatured states could come from the computer simulation and synthetic polymer studies, respectively.) Such an approach is limited, however, since our work with serum albumin shows that the parameters would not generally hold.

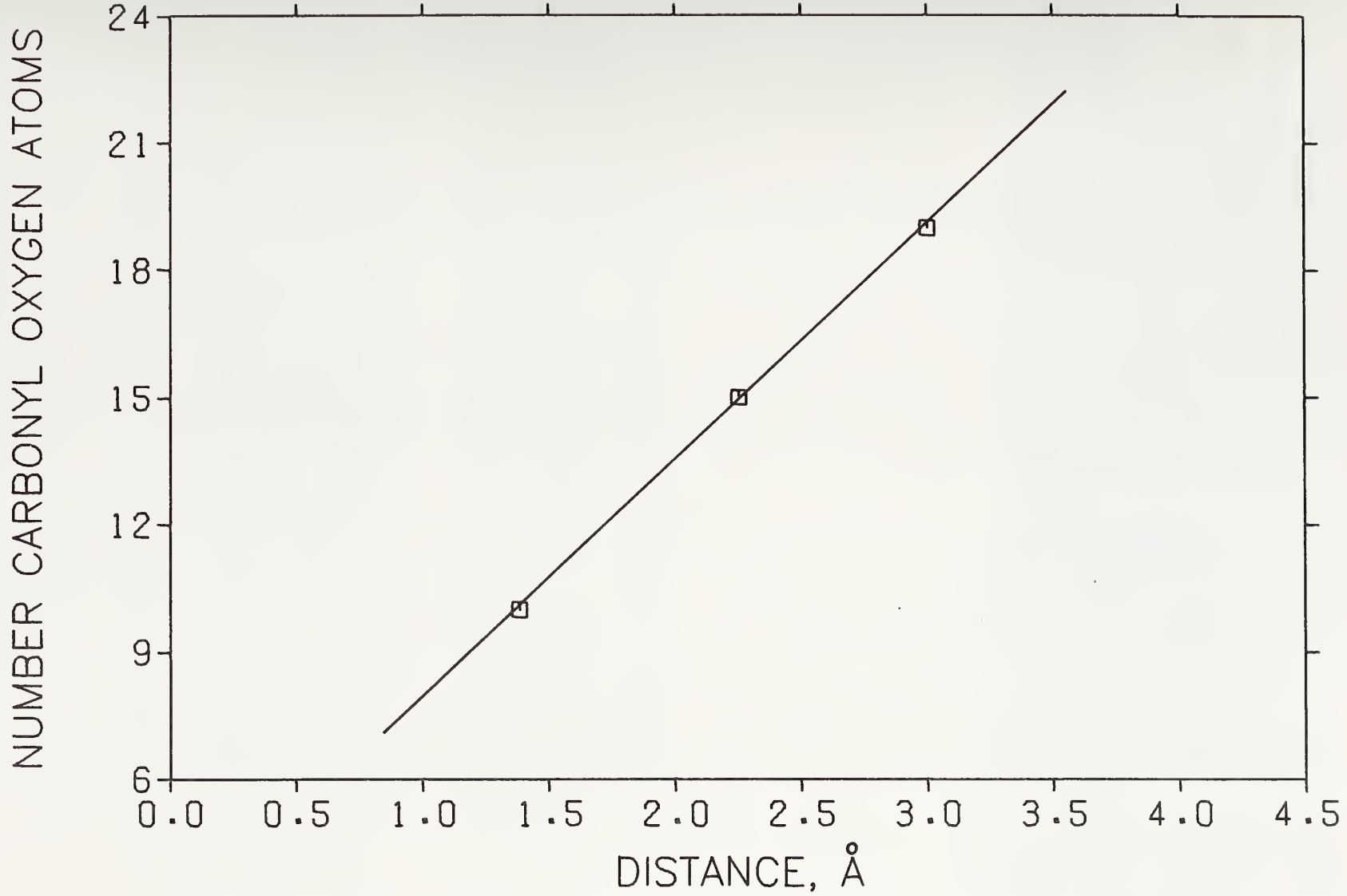

Figure 33: Computer simulation of lysozyme adsorption. Number of carbonyl oxygen atoms vs. distance for the adsorbed conformation yielding the maximum number of carbonyl groups interacting with the surface.

Polyamino Acid Studies - Attempts to determine the bound fraction of a polyamino acid of a known conformation were only partially successful. While the α-helical form of poly-L-lysine (at pD 11.5) and poly-L-glutamic acid (at pD 5.4) adsorbed to yield bound fractions of 0.60 and 0.50 respectively, the random coil form of each polymer did not adsorb or give a difference spectrum. This negative finding may be due to strong polymer-polymer interactions since both polyamino acids are highly charged in their random coil form. The large bound fractions for the α-helical forms is consistent with a bridging mechanism for adsorption. Assuming 3.60 residues per turn of the helix with a rise of 5.38 A, the length of the poly-L-lysine and poly-L-glutamic acid were 1618 and 1180Å, respectively. Studies of polyamino adsorption, with lower molecular weights to avoid bridging, on larger particle size polystyrene latex, to reduce polymer-polymer interactions, are planned.

RESULTS AND DISCUSSION

EFFECT OF ADSORPTION ON THE THROMBIN-FIBRINOGEN REACTION

One method of assessing the effect of adsorption on the thrombin-fibrinogen reaction is to determine the kinetics of fibrinopeptide release for solution compared to adsorbed fibrinogen. Using Finlayson's techniques (20), and using a Sakaguchi assay, we have obtained results for the control experiment where all proteins are in solution. Experiments using fibrinogen insolubilized on Cab-O-Sil have shown no measurable fibrinopeptide release and greatly increased clotting times. Tests have shown that the thrombin in this system is being adsorbed and inactivated. This work will be pursued using a different surface, detergent micelles, which have been shown to enhance the activity of lysozyme.

COMPETITIVE ADSORPTION WITH UNLABELED PROTEINS

Work has been initiated to determine the competitive interactions between blood proteins during the processes of adsorption, displacement, and desorption. By taking advantage of the unique biological properties of fibrinogen and prothrombin, one can study all pairwise combinations of fibrinogen (coagulation with thrombin), prothrombin (isoelectric precipitation at pH 5.3), and serum albumin without introducing any labeling atoms or groups.

The preliminary results obtained thus far show that fibrinogen interacts more strongly than serum albumin with a silica surface. When adsorption proceeds from an initially equimolar solution of fibrinogen and serum albumin (Fig. 34), no albumin is adsorbed. If the initial condition is changed to a 10:1 concentration ratio favoring serum albumin, as in Fig. 35, then some albumin does adsorb, although it appears to be slowly desorbed. Figure 36 shows that adsorbed serum albumin is quickly displaced by fibrinogen in solution. Desorption studies (Fig. 37) show that serum albumin does not desorb at a sufficient rate to account for the rapid displacement by fibrinogen. The conclusion from these studies is that fibrinogen has a much greater affinity for the surface.

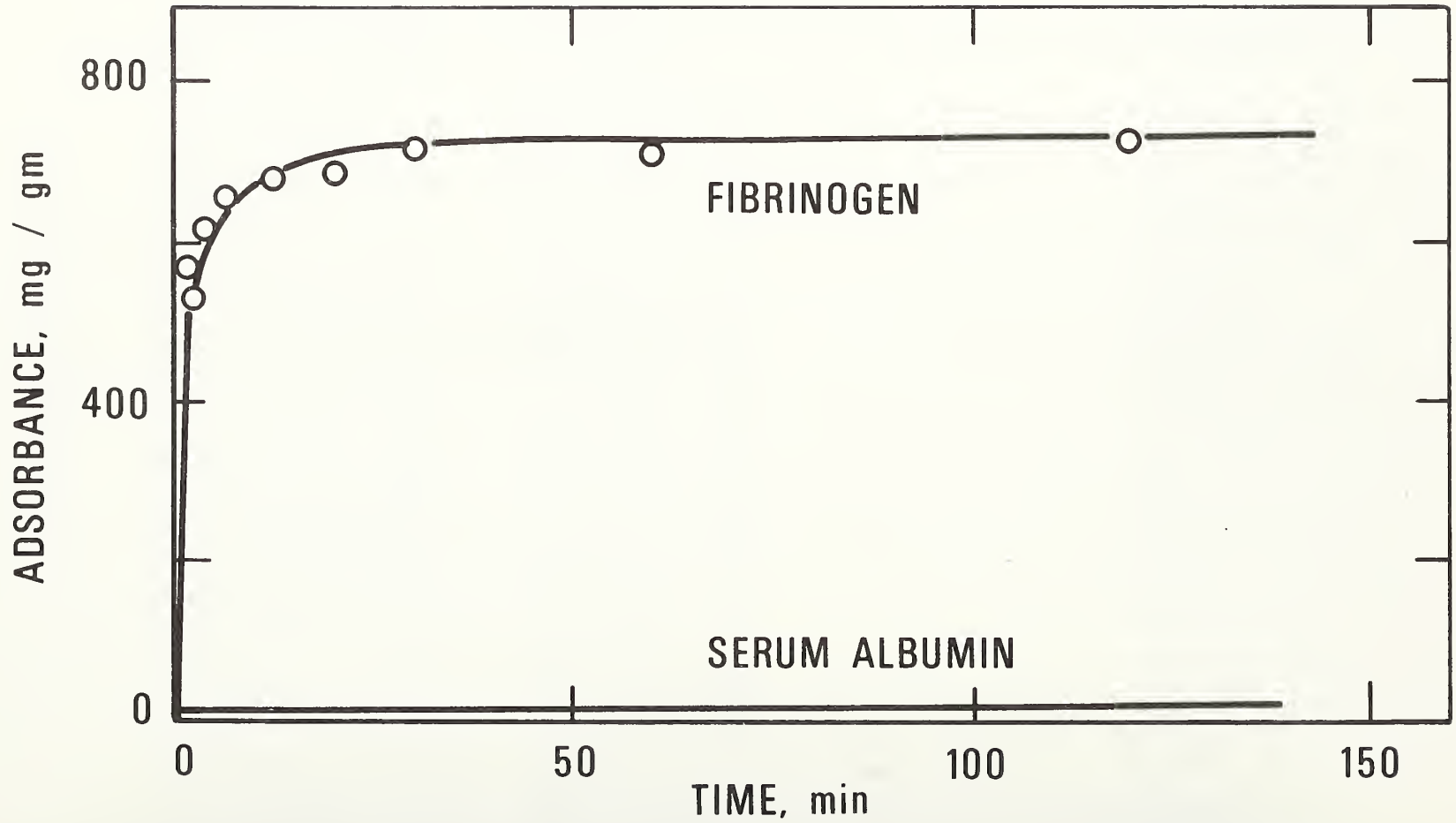

Figure 34: Competitive adsorption of unlabeled serum albumin (1.20 mg/ml) and fibrinogen (6.12 mg/ml).

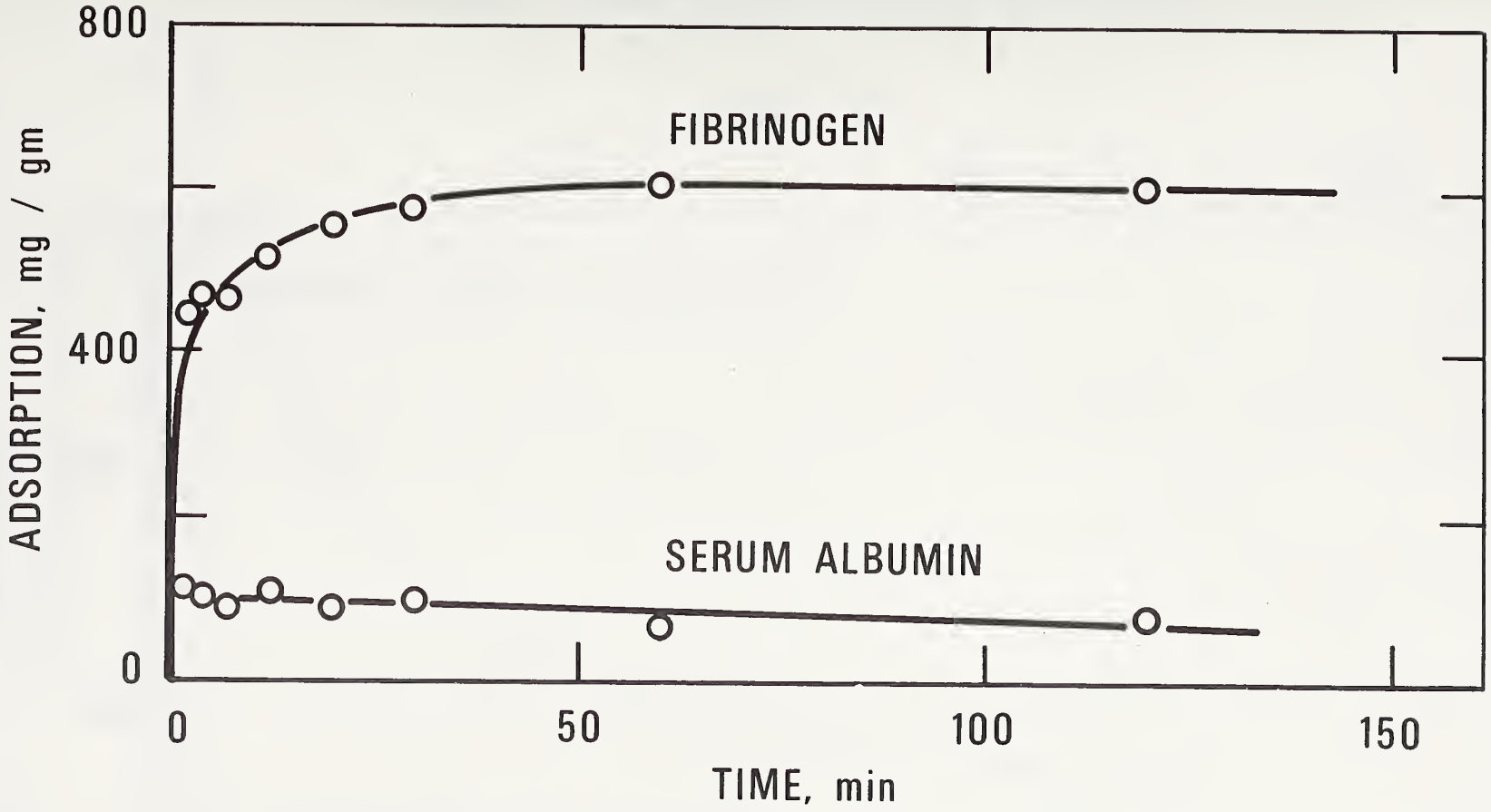

Figure 35: Competitive adsorption of unlabeled serum albumin (5.68 mg/ml) and fibrinogen (3.02 mg/ml).

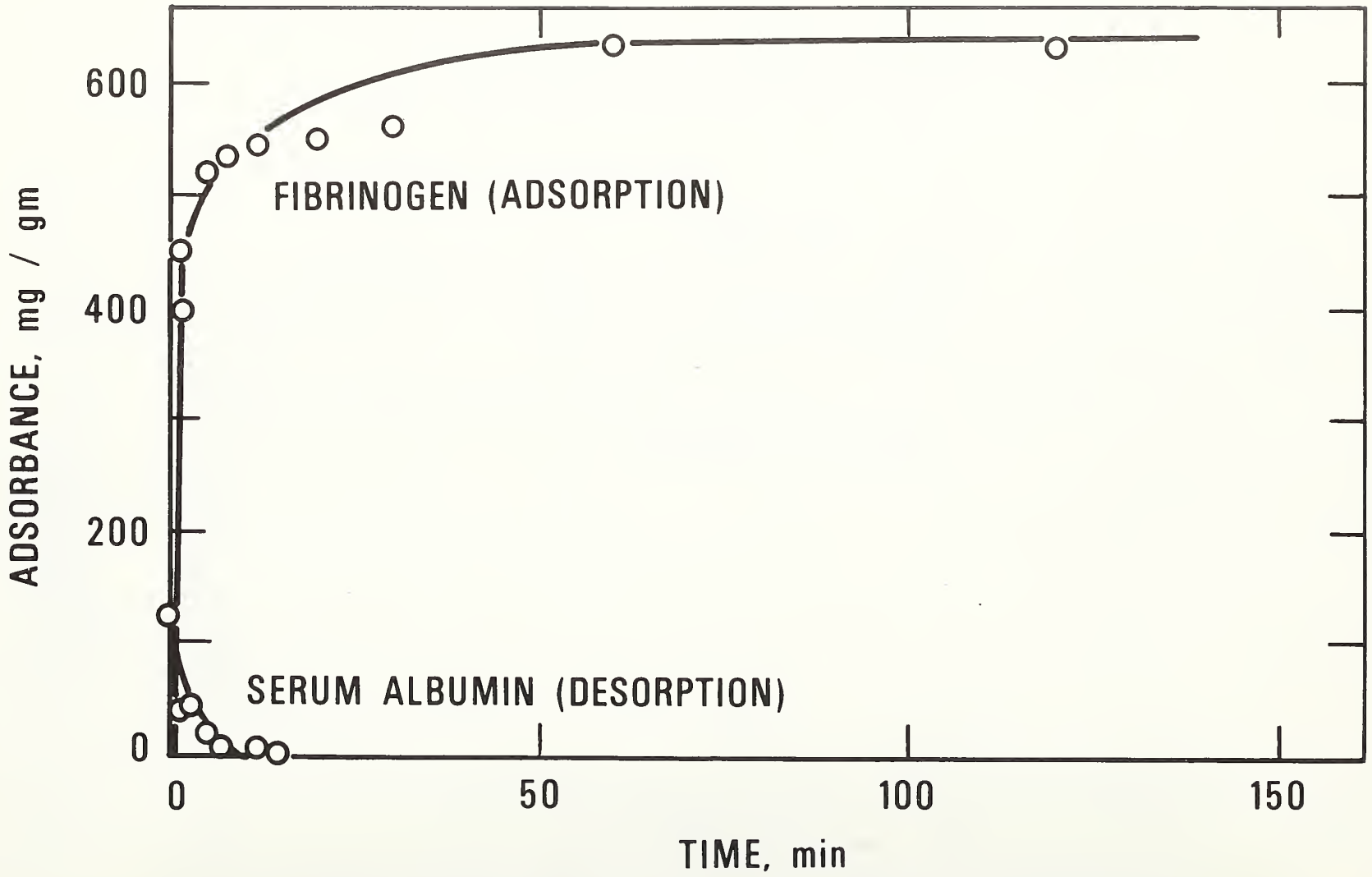

Figure 36: Displacement of serum albumin by fibrinogen

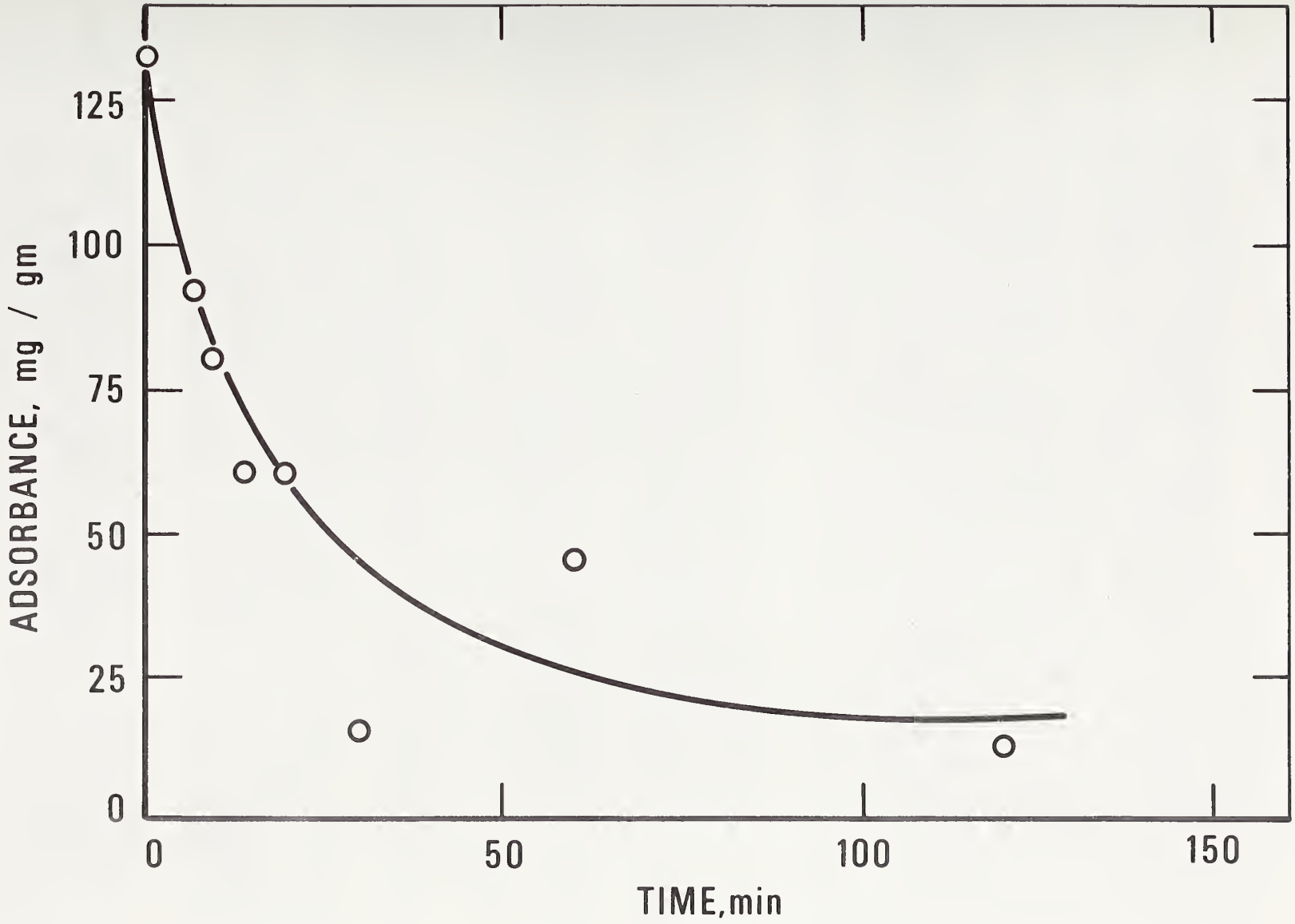

Figure 37: Desorption of serum albumin

RADIOTRACER RATE STUDIES

Development of Washing Technique

The development of an effective washing technique is of primary importance in any adsorption investigation. Washing must be effective in completely removing any clinging solution (carry-out) but should be as gentle and of as short a duration as possible to minimize desorption.

Protein solutions further complicate the problem by their possible formation of a denatured film at the air-solution interface which must be taken into account both in adding and withdrawing sample surfaces. The experimental approach was to first devise a scheme which would avoid artifacts from such a denatured film, even at the expense of a time consuming procedure and a prolonged washing time. Once reproducible results could be obtained from this system, simplifying changes in the procedure could be made with these results as a reference point. The reference technique chosen was to add the protein solution to an adsorption vessel containing the sample slides covered with a layer of water. After the desired adsorption time, the entire vessel was rinsed by overflowing with rinse water.

While this procedure avoids passing slides through the air-solution interface, only one adsorption time could be measured per run and the rinse time was long because of the large volume of protein solution to be diluted. Several simplifing modifications were found to yield results identical to that of the reference technique described above.

1) Slides, contained in 1/8" deep planchets filled with buffer, may be inserted directly into the protein solution. Hydrophilic surfaces wet with water may be directly passed through the albumin solution-air interface. This fact remains to be demonstrated for hydrophobic surfaces.

2) Slides may be removed from the solution by use of the same planchets with the planchets then individually overflowed.

3) Rinsing of slides contained in shallow planchets is generally inefficient as judged by the time taken for a dye solution to be cleared. This is due to the difficulty in obtaining mixing in the thin layer over the top of the slide and from solution trapped between the slide and the planchet. Rinsing is more efficiently done by immersing the planchet into a large rinse vessel, removing the slide from the planchet, and rinsing the slide separately in additional vessels.

4) The hydrophilic samples may be passed through the albumin solution-air interface without irreversibly carrying out a surface film. Rinsing in this case must be somewhat more vigorous but conditions can easily be found that give good agreement with the reference technique. Static rinsing by dipping into vessels filled with water does not appear to be sufficient.

5) A turbulent flow rinse did not give reproducible results. While it did seem to minimize washing time, the degree of turbulence and its distribution over the slide was too difficult to control precisely.

In summary, the simplest technique suitable for hydrophilic surfaces and albumin solutions consists of 3 steps:

1) Albumin solution added to adsorption vessel containing slides covered with thin film of buffer.

2) Slides removed through air-solution interface.

3) Slides rinsed with flowing water.

The rinse conditions we have chosen are 5 seconds in water flowing at a rate of one liter per minute at 37°C. We have not explored the acceptable limits of these parameters but only moderate adherence to these conditions has given good reproducibility.

Surface Roughness Determinations

The weight of protein adsorbed per unit area is calculated using the geometrical area of the counting mask. In order to make meaningful comparisons of adsorbance on different materials, some measure of the actual available surface area of the samples must be used. We have measured the adsorbance of two small molecules, stearic acid and hexadecane, on the various surfaces and attributed the differences between the surfaces as due to surface roughness. Similar methods have previously been used as measures of surface area. For example, Timmons, Patterson, and Lockhart (71) measured the adsorbance of stearic acid on iron with different polishing treatments. Surface areas were then calculated by comparing these results with that found on fire polished glass which was assumed to be perfectly smooth.

Both stearic acid $-^{14}C$ and hexadecane $-^{14}C$ were adsorbed overnight from benzene solution onto quartz, chromium, and platinum slides. Aliquots of both solutions were deposited on clean samples of the three materials to evaluate the relative backscatter efficiency. Relative adsorbance values were used to calculate roughness factors as shown below.

Relative Roughness Factors

	Platinum	Quartz	Chrome
hexadecane	1.00	1.19	1.36
stearic acid	1.00	1.33	1.38
average	1.00	1.26	1.37

The fact that the roughness factors calculated using the non-polar hexadecane and the polar stearic acid are in good agreement, supports the use of such factors in comparing the various surfaces. However, as such factors are only relative to the smoothest surface measured and do not reflect the absolute area available to a protein molecule, the data presented here have not had such factors applied to them. They may be kept in mind, but the corrections are relatively small.

Adsorption rate results

Chromium

All results were obtained using the flowing rinse procedure outlined in the previous section. Figure 38 shows adsorption rates for albumin on chrome at two different concentrations. Each point is an average of three samples. The concentration isotherm is flat between concentrations of 2.5 and 5.0 mg/ml so 3 mg/ml is apparently on the plateau while 0.52 mg/ml is considerably below. The reaction rates vary markedly with concentration with the rate of the more concentrated solution almost too fast for our technique to measure. The "equilibrium" adsorbance of 1.7 mg/m^2 of the higher concentration compares favorably with the value of 1.5 mg/m^2 found by ellipsometry as shown in Figure 39. As the ellipsometric adsorbance is determined in situ, this agreement reinforces the contention that our rinse procedure is not desorbing appreciable amounts of protein. The apparent difference in rate is due to the lack of stirring in the ellipsometer cell.

Silica

Figure 40 shows the adsorption isotherm of albumin on fused silica and Figure 41 gives the adsorption rate for a solution concentration on the plateau of the isotherm. A semi-logarithmic plot has been used for the rate data to expand the short time period. The adsorption rate on silica is slower than that on the chrome; apparent equilibrium adsorbance is not reached for 10 minutes compared to 10 second for chrome. Additional data at the plateau adsorbance will be collected in the course of desorption experiments currently being carried out.

Platinum

Preliminary adsorption measurements on platinum gave equilibrium adsorbance values of 40 - 50 mg/m^2. Such high results prompted a search for artifacts in connection with this surface. Two possible sources, backscatter efficiency and surface roughness, have already been mentioned and the correction factors were much too small to account for the data. A third possibility, specific adsorption, was tested and appears to be the responsible phenomenon.

Specific ion adsorption was tested by immersing chromium, quartz, and platinum slides in a NaI^{131} solution for 18 hours. The slides were then rinsed, dried, and counted. Both the chrome and quartz showed

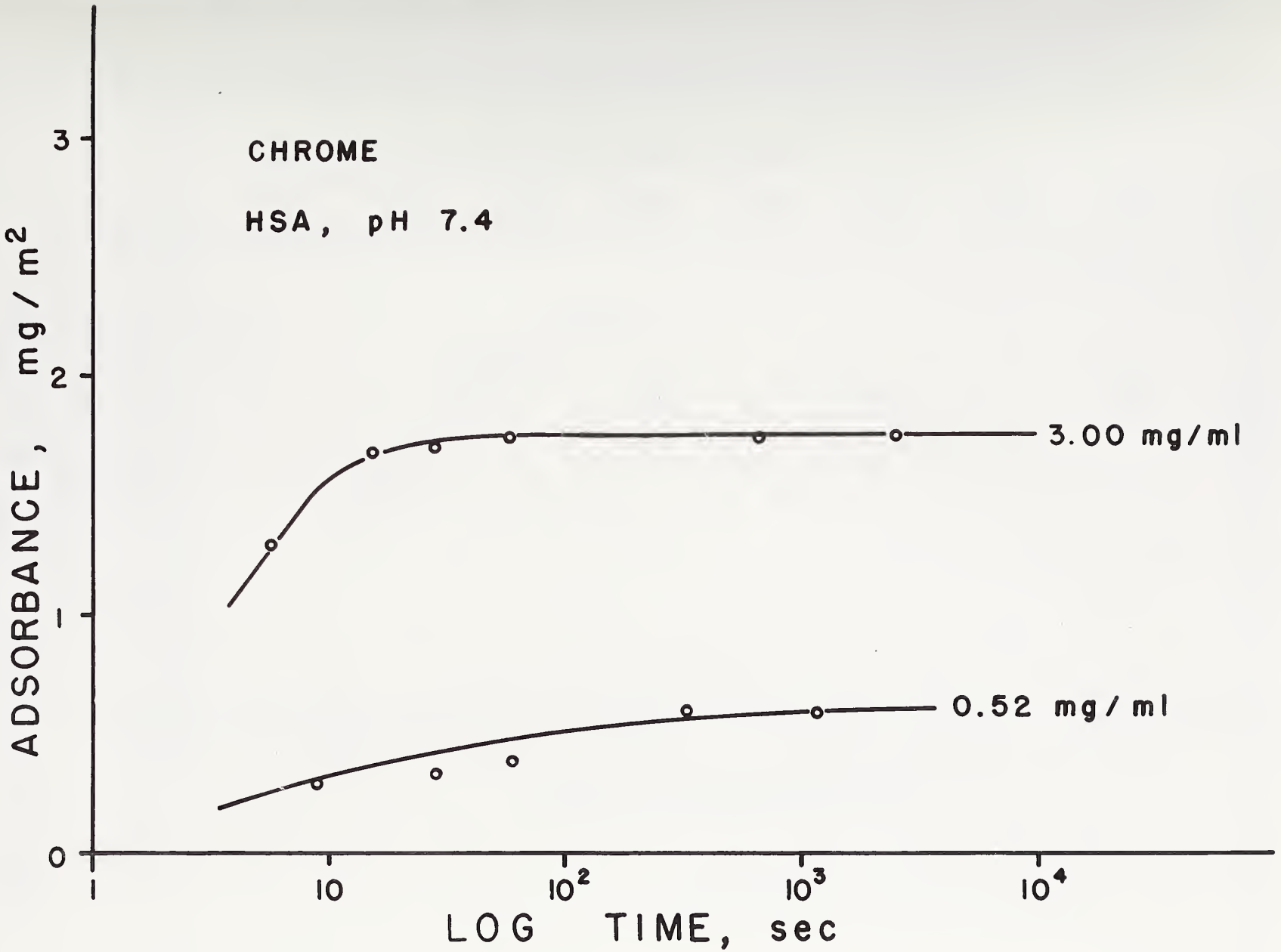

Figure 38: Rate of albumin adsorption on chromium

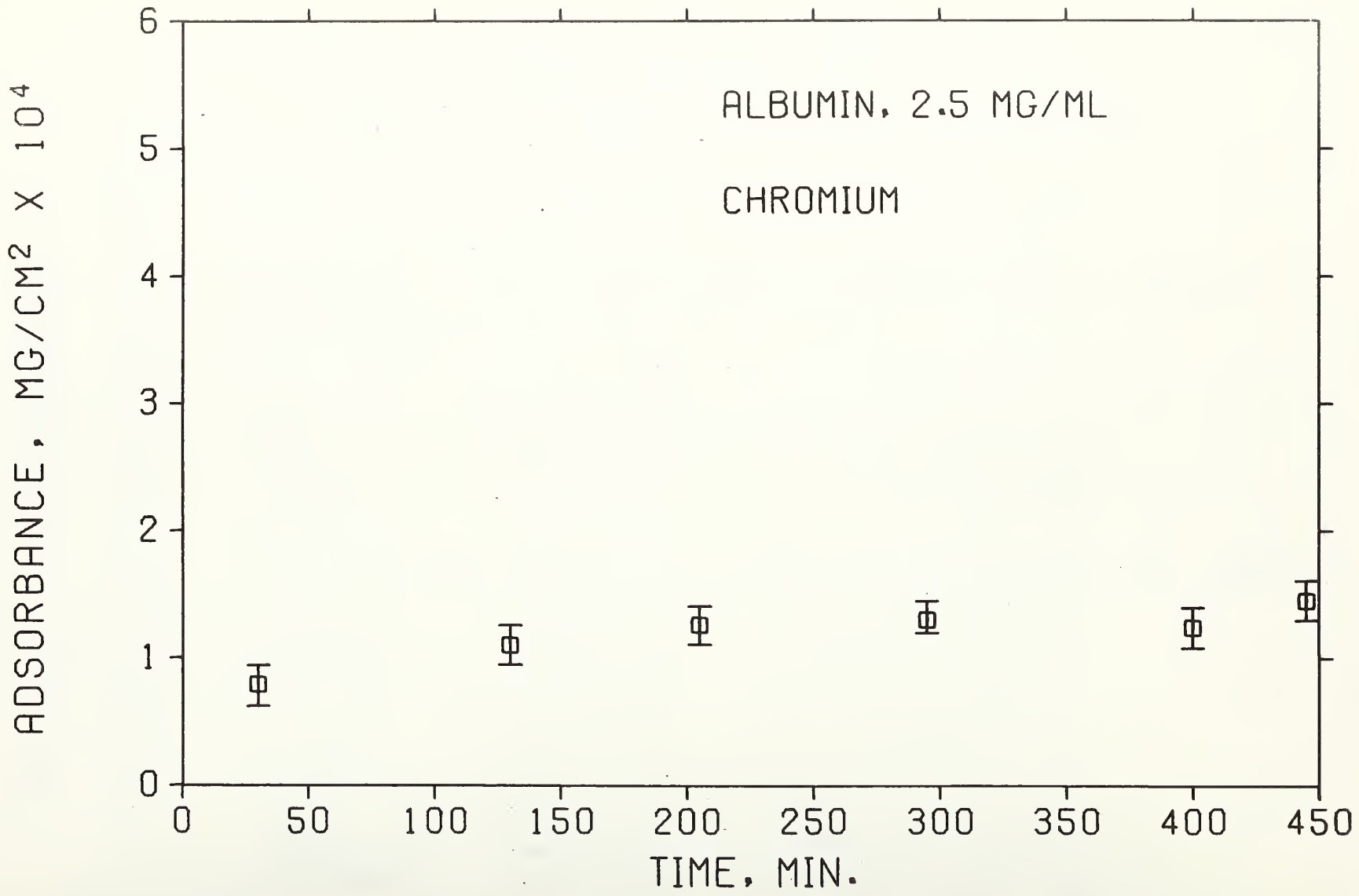

Figure 39: Adsorbance of albumin on chromium by ellipsometry

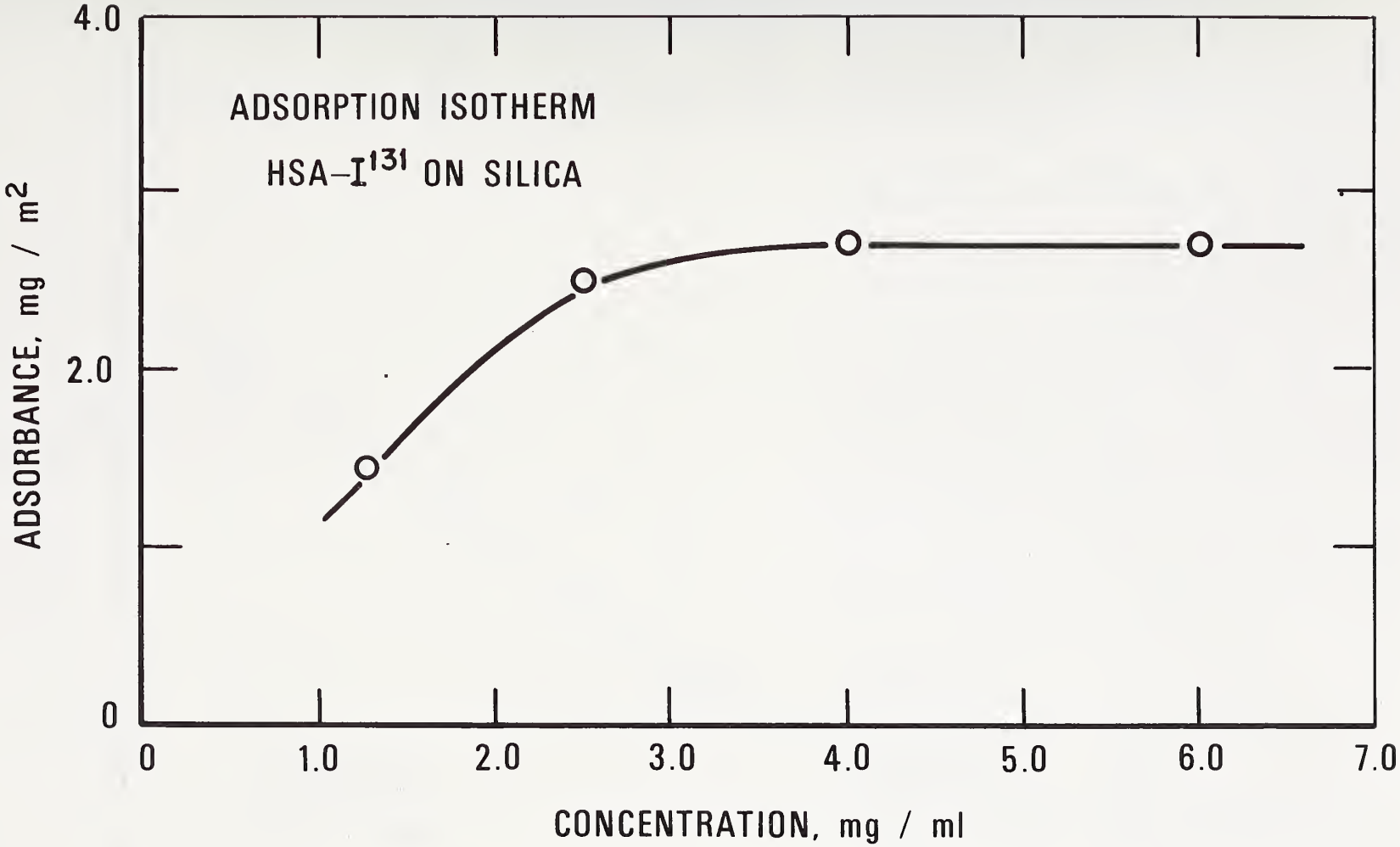

Figure 40: Adsorption isotherm of albumin on silica

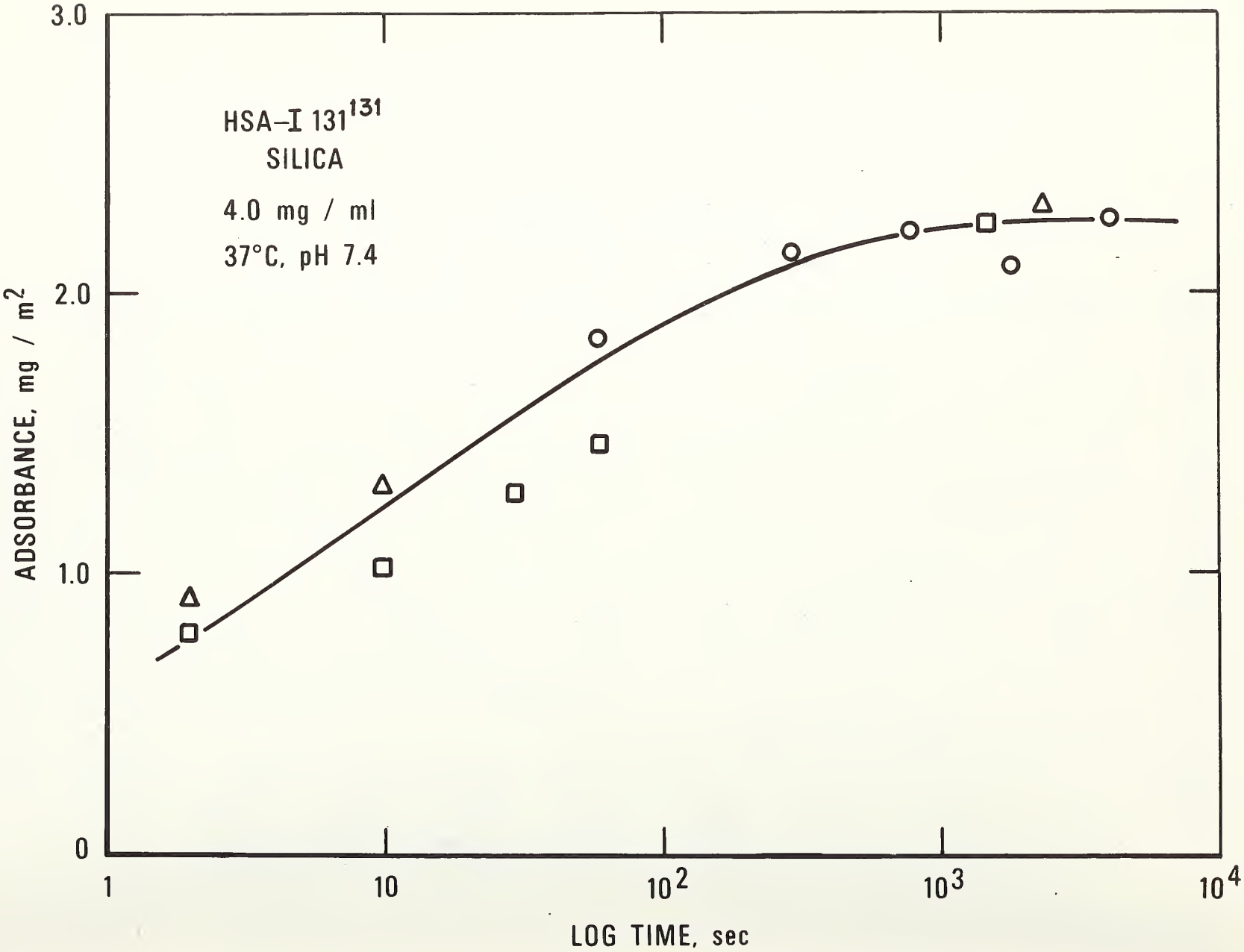

Figure 41: Rate of albumin adsorption on silica

very small iodide ion adsorbance while the platinum was over 900 times higher. To test whether this specific ion adsorption could cause enhanced adsorption of iodine labeled albumin, two labeled albumin solutions with greatly different specific activities were made up and used for adsorption measurements on platinum. In the absence of any enhanced adsorption, the two different activity samples would give the same result. A preferentially adsorbing species would, however, be present in very different concentrations in the two solutions and the labeled to unlabeled ratios in the two adsorbed films could well be different. As the experimental results differed by almost a factor of two for the two solutions, it is evident that the labeling ratio on the surface is not the same as in solution and that this difference causes an overestimation of adsorbance on this surface.

The only solution to this problem is albumin with another label, perhaps ^{14}C or ^{3}H which would not introduce another artifact. Although platinum would be useful as a reference material, it was not considered important enough to the program to warrant such an effort and platinum has been dropped from the radiotracer program. The specific adsorption noted with this material serves, however, as a warning of the possible artifacts involved in the use of radiolabeled proteins and the other surfaces will also be checked for this effect.

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